CHALLENGES FACING FOODBORNE MYCOTOXIN REGULATION AND PUBLIC HEALTH CONSEQUENCES OF EXPOSURE IN CHILDREN UNDER 5 YEARS OF AGE

By

Denis Male

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Food Science ─ Environmental Toxicology — Doctor of Philosophy

2017

ABSTRACT

CHALLENGES FACING FOODBORNE MYCOTOXIN REGULATION AND PUBLIC HEALTH CONSEQUENCES OF EXPOSURE IN CHILDREN UNDER 5 YEARS OF AGE

By

Denis Male

Foodborne mycotoxins are low molecular weight secondary metabolites produced by fungi and commonly co-occur in staple crops such as wheat, barley, and corn. Hence, they jointly cause adverse effects including decreased growth and immune system dysfunction. However, there is limited information on their relative toxicities which makes risk assessment difficult. As a result, current mycotoxin regulations are mostly based on individual toxin studies conducted using animal models and in some instances, supplemented with very limited epidemiological data. This dissertation presents aflatoxin exposure data of Ugandan children aged 1-5 years and also presents relative toxicities of trichothecenes that commonly co-occur in food.

To assess the level of aflatoxin exposure among the children, we conducted a cross sectional survey in three regions (chapter 2). We used isotope dilution mass spectrometry to determine the concentration of aflatoxin B_1-lysine adducts in their serum. We found detectable concentrations of these bioimarkers in all samples that were tested. We observed differences in aflatoxin exposure among regions, but there were no significant differences in exposure by age group, sex, and residence type. The levels of aflatoxin exposure among Ugandan children are consistent with concentrations associated with growth impairment in other epidemiological studies.

Using case studies of aflatoxin and cassava cyanide, we discussed the heightened risk of particular diseases from consuming monotonous diets based on maize, groundnuts, and cassava

(chapter 3). We discussed the potential role of increased dietary diversity in counteracting these diseases. Studies show that, increased dietary diversity can reduce consumption of toxins and increase intake of nutrients that could counteract the toxicity of such chemicals. Because interventions focused solely on reducing toxin levels have proven difficult to sustain, we suggest that future food policy reforms should take into consideration the multifaceted benefits associated with improved dietary diversity to promote health.

To address the issue of co-occurrence of in food, we proposed assigning of toxic equivalency factors (TEFs) to mycotoxins that have similarities in their mode of action. Here, we employed a mouse model to compare the oral exposure anorectic potencies of simple trichothecenes using the US Environmental Protection Agency (US-EPA) benchmark dose (BMD) method and the incremental area under the curve (IAUC) method (chapter 4). The relative potencies of these toxins were calculated as the ratios of their BMDs (or IAUC) to that of DON. The rank orders for anorectic potency were: DON ≈ 3-ADON ≈ 15-ADON < NIV < HT-2 < FUS-X << T-2 and DON < 3-ADON ≈ 15-ADON < NIV < FUS-X << T-2 < HT-2 for the BMD and IAUC method, respectively. We also used a mink emesis model and the BMD method to compare the toxicities of the trichothecenes administered via gavage and intraperitoneally (chapter 5). Our results showed that mink were more sensitive to orally administered toxins than to toxins administered by IP. The rank order for the oral emetic potency was: HT-2 ≈ T-2 > FX > DON > 15-ADON > NIV > 3-ADON. Taken together, the results presented in this dissertation demonstrate that assigning of TEFs for trichothecenes that co-occur in food is possible.

This dissertation is dedicated to my late parents, my wife Erica Male and children Timothy, Crystal and Joseph. Thank you so much for making life more purposeful.

ACKNOWLEDGMENTS

My deep gratitude goes first to my major advisor and mentor Dr. Felicia Wu, who expertly guided me through my graduate education. I would like to also thank my committee members Dr. James Pestka, Dr. John Linz, and Dr. Edward Walker for their tremendous support. The work presented in this dissertation is a testimony of their invaluable advice and dedication.

I gratefully acknowledge the United States Agency for International Development (USAID), through the Borlaug Higher Education in Agricultural Development (BHEARD) scholarship program for funding my PhD. I sincerely thank Dr. Fred Derksen for giving me this unique opportunity and for having faith in me.

Many thanks to my colleagues at Michigan State University: Jina Yu, Nana Baah Pepra-Ameyaw, and Isaac Dramadri for your moral support. Special thanks to Dr. Wenda Wu (Pestka Lab) for generating data from the mink and murine feeding studies and Dr. Nicole Mitchell who taught me a great deal about scientific research and life as a PhD student.

I gratefully appreciate the team in Uganda for supporting me during my fieldwork activities – Dr. Monica Etima, Sr. Alice Elwana, Dr. Vincent Muwanika Dr. Dorothy Nakimbugwe, Prof. Archileo Kaaya, and the staff of the various hospitals where samples were collected.

Finally, I would like to thank my loving wife Erica, my children Timothy, Crystal and Joseph, the Dietz family, and my friends for their unwavering love and support during my PhD journey. In fair honesty, it is impossible to mention everyone. But to those I didn't mention, thank you and may you be blessed with only the best in your lives.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

5-HT	5-Hydroxytryptamine
A.	*Aspergillus*
ADON	Acetyldeoxynivalenol
AFB_1	Aflatoxin B_1
ARfD	Acute Reference Dose
AT	Adenine-thymine
ATA	Alimentary toxic aleukia
AUC	Area under the curve
BIRB	Biomedical Institutional Review Board
BMD	Benchmark dose
BMDL	Benchmark dose lower bound
BMDS	Benchmark Dose Software
BMI	Body Mass Index
BMR	Benchmark Response
C	Carbon
CD	Cluster of Differentiation
CTLA4	Cytotoxic T-lymphocyte associated protein 4
CTZ	Chemoreceptor trigger zone
CYP	Cytochrome P450
DAS	Diacetoxyscirpenol
DNA	Deoxyribonucleic acid

DON	Deoxynivalenol
EC	European Commission
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
F.	*Fusarium*
FAO	Food and Agricultural Organization
FAPY	Formamidopyrimidine
FB	Fumonisin B
FCS	Flow Cytometry Standard
FDA	United States Food and Drug Administration
FX	Fusarenon-X
GIT	Gastrointestinal tract
GPI	Glycosylphosphatidylinositol
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
HT-2	HT-2 toxin
IACUC	Institutional Animal Care and Use Committee

IARC	International Agency for Research on Cancer
IAUC	Incremental area under the curve
IDMS	Isotope dilution mass spectrometry
IFN	Interferon
IgA	Immunoglobulin A
IL	Interleukin
IP	Intraperitoneal
IRB	Institutional Review Board
JECFA	Joint FAO/WHO Expert Committee on Food Additives
KBD	Kashin-Beck disease
KW	Kruskal-Wallis
LAZ	Length-for-Age Z-score
LD	Lethal dose
LOAEL	Lowest Observed Adverse Effect Level
LOD	Limit of detection
LOEL	Lowest Observed Effect Level
MAPK	Mitogen-activated protein kinase
MSU	Michigan State University
MWW	Mann-Whitney-Wilcoxon
NIV	Nivalenol
NK	Natural killer
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level

OH	Hydroxyl
OTA	Ochratoxin A
PAMP	Pathogen associated molecular pattern
PCB	Polychlorinated biphenyls
PCDD	Polychlorinated dibenzodioxins
PCDF	Polychlorinated dibenzofurans
PD1	Programmed cell death protein 1
PMTDI	Provisional Maximum Tolerable Daily Intake
PRR	Pathogen recognition receptor
PYY	Peptide YY
RDT	Rapid diagnostic test
SA-PE	Streptavidin-phycoerythrin
SCF	Scientific Committee on Food
spp	species
STAT6	Signal transducer and activator of transcription
T-2	T-2 toxin
TCDD	2,3,7,8-tetrachlorodibenzodioxin
TDI	Tolerable Daily Intake
TEF	Toxic equivalency factor
TEQ	Toxic Equivalence Quotient
TGFβ	Transforming growth factor beta
TLR	Toll-like receptor
TNF	Tumor necrosis factor

TRP	Transient receptor potential
UCM	Uncomplicated malaria
UNCST	Uganda National Council for Science and Technology
UPLC	Ultra Performance Liquid Chromatography
US-EPA	United States Environmental Protection Agency
USSR	Union of Soviet Socialist Republics
WAZ	Weight-for-Age Z-score
WHO	World Health Organization
WLZ	Weight-for-Length Z-score
ZEA	Zearalenone

CHAPTER ONE: Background

1. Major food borne mycotoxins of public health significance

Mycotoxins are low molecular weight natural products produced as secondary metabolites by fungi in pre-harvested crops, harvested produce or their products and are capable of causing harmful effects in plants and animals (Bennett and Inamdar, 2015). The term mycotoxin was coined in 1962 following a mysterious and disastrous turkey X disease that killed over 100,000 turkeys in England. It was later discovered to have been caused by consumption of aflatoxin-contaminated peanut-based feed (Bradburn *et al.*, 1994; Wogan *et al.*, 2012; Zain, 2011). These toxins contaminate approximately 25% of all cereals produced worldwide which makes them one of the biggest threats to food security globally (Turner, 2013). The most commonly contaminated crops include food staples such as maize, groundnuts, wheat, barley, oats, and sorghum, which make exposure continuous in some communities. Despite having the highest exposure, mycotoxins are largely neglected in most developing countries because, the perceived benefit of interventions is lower than that of other public health issues such as infectious diseases e.g. malaria control (Wild and Gong, 2010). As a consequence, the health burden due to mycotoxins is disproportionately distributed and is highest among the low income nations. The most agro-economically important types of mycotoxins that contaminate foods worldwide include aflatoxins, trichothecenes, fumonisins, ochratoxins, and zearalenone (Zain, 2011).

1.1 Aflatoxins

Aflatoxins are a group of highly substituted coumarins containing a fused dihydrofurofuran moiety (Fig. 1), that are produced by members of the *Aspergillus spp* (Turner, 2013). There are four types of aflatoxins that naturally occur in foods. These include aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2). Putatively, *A. flavus*

produces AFB_1 and AFB_2 while *A. parasiticus* produces both B- and G-type aflatoxins (Udomkun *et al.*, 2017). Aflatoxin B_1 is the most biologically potent and so far the most studied mycotoxin due to its toxic and genotoxic potency (Van Egmond *et al.*, 2007; Wu *et al.*, 2014b).

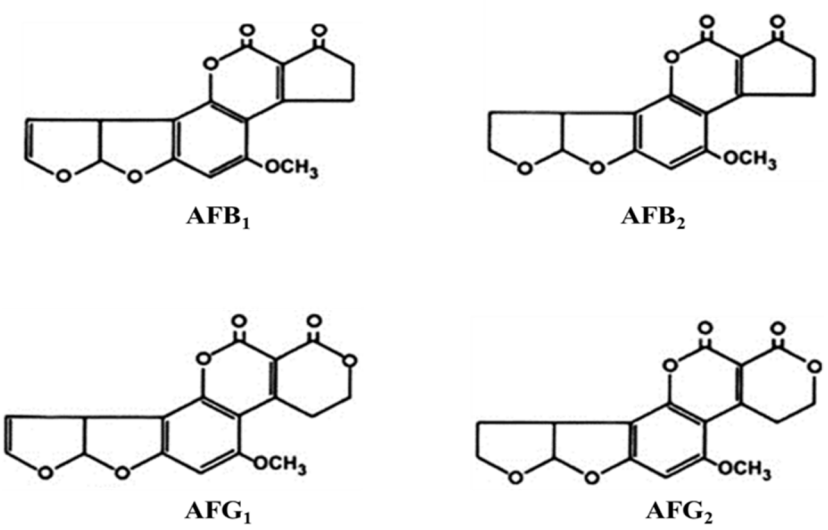


Figure 1. Structure of naturally occurring aflatoxins (Quiles *et al.*, 2015).

Aflatoxins contaminate common crops and foodstuffs including maize, peanuts, cottonseed, wheat, barley, cocoa, beans, rice, copra, dried fruits, spices, figs, and crude vegetable oils (Karlovsky *et al.*, 2016). Maize and groundnuts are the most contaminated but also among the most frequently consumed staples in high risk regions of Sub-Saharan Africa and Asia (Hell and Mutegi, 2011; Wu *et al.*, 2013a). Once they are produced in food, aflatoxins remain stable under normal handling, processing, and storage conditions (Inoue *et al.*, 2013). Processing methods including heating, fermentation, and chemical treatment may, to varying extents reduce AFB_1 in food products (Karlovsky *et al.*, 2016). Thermal processing treatments such as boiling, roasting, frying, and nixtamalization, may degrade AFB_1 in foods although the reductions vary depending on the type of thermal treatment as well as the food matrix in which the toxin is contained. For instance, in a previous study, boiling and frying of maize grits resulted in 28% and 34-53%

reductions in mean AFB_1 concentrations, respectively (Kabak, 2009). Moreover, Raters and colleagues found that, a 30 min 150˚C treatment of spiked samples caused complete AFB_1 degradation in protein matrices as compared to 50% achieved in carbohydrate matrices (Raters and Matissek, 2008). Brewing yeasts such as *S. cerevisiae* and *S. pastorianus* yeast may adsorb and degrade AFB_1 during the fermentation process. In a recent study, the levels of AFB_1 were decreased by 70% in wine fermentations but remained unchanged in beer fermentations (Inoue *et al.*, 2013). The available evidence thus illustrates that, the partial efficacy of most aflatoxin detoxification and elimination methods means that contamination of foods certainly results in human exposure to this highly toxic carcinogen. Previous reports have estimated that about 4.5 billion people worldwide are exposed to aflatoxins (Wild and Gong, 2010). Therefore there is a great need to pay more attention to aflatoxins to minimize their impact on public health.

1.1.1 Metabolism of aflatoxins in the human liver

Aflatoxins are lipophilic and once ingested, they are rapidly absorbed across the luminal membrane of the mammalian small intestine (Ramos and Hernandez, 1996). The majority are distributed to the liver and to a lesser extent to kidneys and lungs (Bren *et al.*, 2014; Dohnal *et al.*, 2014). Cytochrome P450 (CYP) enzymes including CYP 3A4, CYP 3A5, and CYP 3A7 biotransform AFB_1 in the liver to mainly AFB_1-8,9- endo epoxide and the genotoxic AFB_1-8,9-exo epoxide. These enzymes may also yield a small amount of AFQ_1. The reactions catalyzed by CYP 1A2 result in oxidation of AFB_1 to predominantly AFB_1-8,9- endo epoxide, AFM_1 and AFQ_1 (Bren *et al.*, 2014; Turner, 2013; Turner *et al.*, 2007) although previous reports indicate that CYP 1A2 may also generate some exo-8,9-epoxide (Bbosa *et al.*, 2013; Guengerich *et al.*, 1998). In addition to CYP450s, liver lipoxygenases may also catalyze the transformation of AFB_1 to AFB_1-8,9-epoxides (Dohnal *et al.*, 2014; Liu and Massey, 1992).

Most adverse effects of AFB_1 are attributed to the formation of AFB_1-8,9-exo epoxide, which is genotoxic since it covalently binds with DNA to form the 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 (AFB_1-N^7-guanine) adduct (Dohnal *et al.*, 2014; Wu *et al.*, 2013b). AFB_1 can also be hydroxylated to AFM_1, a metabolite known to contaminate breast milk and is also excreted in urine. There is increasing interest in the toxicity of AFM_1 since it can be epoxidized to AFM_1-8.9-epoxide which is capable of binding to DNA to form AFM_1-N^7-guanine adduct (Jager *et al.*, 2011). Moreover, the role of this adduct in hepatocellular carcinoma is not fully elucidated. The other metabolites of AFB_1 such as AFQ_1, AFP_1, aflatoxicol, and other naturally occurring aflatoxins such as AFB_2, G_1, and G_2, are not efficiently epoxidized and therefore non-genotoxic and less toxic than AFB_1 (Wild and Turner, 2002).

The primary pathway for AFB_1-8,9-epoxide detoxification is through conjugation with glutathione (Xu *et al.*), catalyzed by glutathione-S-transferase (GST) enzymes to form aflatoxin-mercapturic acid which is then excreted in urine (Turner, 2013). The rate of AFB_1 epoxide formation versus the rate of conjugation with glutathione influences the sensitivity of individuals to the effects of aflatoxin exposure (Dohnal *et al.*, 2014). The unabsorbed AFB_1 as well as the biotransformed metabolites are ultimately excreted in urine, feces, and breast milk.

1.1.2 Biomarkers of aflatoxin exposure

One of the critical and challenging steps of epidemiological risk assessment is estimating exposure. The traditional approaches that are based on quantitating intake of contaminated food are unreliable due to heterogeneity in distribution of mycotoxins in foods (Turner 2013). Although still under development and require extensive validation, molecular biomarkers provide a more accurate and consistent estimation of human exposure to mycotoxins. Biomarkers are defined as objective chemical, physical and biological measurements that reflect

the interaction between a biological system and a potential hazard. They may be used as surrogate endpoints for certain clinical outcomes of pathology or pharmacological interventions (Strimbu and Tavel, 2010). For purposes of biomonitoring, biomarkers may be classified into three major groups i.e. biomarkers of exposure, biomarkers of effect, and biomarkers of susceptibility. The biomarkers of exposure may be used to determine internal dose and to derive external exposure. Biomarkers of effect are used in hazard identification and in dose-response assessments to determine the relationship between exposure and a putative adverse effect of an agent. Biomarkers of susceptibility may be used to determine the inherent sensitivity of an individual to toxic effects of the substance or toxic agent (Nordberg, 2010; WHO, 1993).

For AFB_1 exposure, there are a variety of macromolecules including AFB_1, AFB_1-N^7-guanine, AFM_1, and AFB_1-lysine can be used as biomarkers and these have been extensively studied and validated using epidemiological studies conducted among different populations especially in sub Saharan Africa and South-East Asia (Gambacorta *et al.*, 2013; Groopman *et al.*, 1993; Scholl *et al.*, 1995). Various methods have been developed to quantitatively determine macromolecules. Notably, the HPLC for AFM_1 in milk (Bellio *et al.*, 2016), ELISA for AFB_1-albumin, and the IDMS for AFB_1-lysine adducts in serum (Scholl *et al.*, 2006). Past studies have reported that the concentrations of AFM_1, AFB_1-N^7-guanine, AFB_1-albumin and AFB_1-lysine adducts correlate with doses of AFB_1 ingested (Scholl *et al.*, 2006; Turner, 2013). The AFB_1-N^7-guanine adducts are chemically very unstable thus**,** can be reliably measured in urine during the initial 24 hours after ingestion of an aflatoxin-contaminated meal. Remarkably, the AFB_1-N^7-guanine adduct is correlated with the risk of HCC (Jager *et al.*, 2011). Approximately 2 percent of all ingested AFB_1 is covalently bound to albumin to form stable AFB_1-albumin adducts ─ studies have reported that no significant decreases in concentration of albumin adducts occurred after 20 years

of storage at -80˚C (Scholl and Groopman, 2008). Because the amount of aflatoxins that covalently bind to albumin is correlated to those that bind to DNA (Groopman *et al.*, 1994), both AFB1-albumin and AFB1-N7-guanine biomarkers can be used to infer HCC risk. More biomarkers for AFB_1 exposure are being explored. For example, Valencia-Quintana and coworkers proposed the use of micro-RNAs, including miR-27a, miR27b, miR-122, and miR-429 as potential indicators for onset of exposure to aflatoxins (Valencia-Quintana *et al.*, 2015). Additionally, human hair has been proposed as an alternative matrix for long term exposure studies although there is limited data to support its significance (Mupunga *et al.*, 2016). However, their application needs to be validated in future epidemiological studies.

1.1.3 Aflatoxicosis in humans

Aflatoxicosis refers to poisoning caused by consumption of foods contaminated with aflatoxins. The susceptibility of individuals to adverse effects of aflatoxin exposure may vary with age, sex, and nutritional status. In addition, aflatoxicosis may be regarded as either severe acute if one is exposed to large amounts over a short time or chronic and sub-symptomatic in instances where low dose exposure occurs over an extended period (Williams *et al.*, 2004). The clinical symptoms of acute aflatoxicosis include anorexia, vomiting, lethargy, fever, jaundice, edema of the legs, abdominal pain, hemorrhagic necrosis of the liver, and death (Dhanasekaran *et al.*, 2011). Indeed, consumption of greater than 1 mg per day of aflatoxin B_1 may cause death in adults (Wild and Gong, 2010). The worst documented aflatoxicosis outbreak in history occurred between January and June 2004 in Kenya, and resulted in 125 deaths. Consequently, a case-control study involving 40 aflatoxin patients and 80 control individuals found that the maize sampled from the patients' households had a significantly higher level of AFB_1 than the one sampled from control households (Patients, 354 ppb; Controls, 44 ppb; $p = 0.004$). Furthermore,

the mean concentration of AFB_1-lysine adducts in serum of patients was higher than that in controls (i.e. Patients, 1200 pg/mg albumin; Controls, 150 pg/mg albumin; $p < 0.0001$) (Azziz-Baumgartner *et al.*, 2005). Thus, concluding that maize was the most likely source of AFB_1 that was implicated in the etiology of the observed maladies.

1.1.4 Immunosuppressive effects of AFB_1

The mechanism of AFB_1 induced immunomodulation is via the binding of the highly reactive but unstable AFB_1-8,9-epoxides with DNA, RNA, and proteins. The binding directly or indirectly inhibit production of cytokines, chemokines, and enzymes resulting in alteration of immune system function (Mohsenzadeh *et al.*, 2016). Chronic exposure is associated with increased susceptibility to infections in humans. An epidemiological study among adult Ghanaians (19-86 years) found that high serum levels of AFB_1 (> 0.9068 pmol/mg albumin) was associated with significantly lower percentages of activated B- and T-lymphocytes as well as lower perforin and granzyme-A expression by cytotoxic T cells (Jiang *et al.*, 2005). In addition, AFB_1 can change proliferation and differentiation of intestinal epithelial cells that form a natural barrier to infections in the GIT and also produce antimicrobial peptides and enzymes (Mohsenzadeh *et al.*, 2016). The presence of detectable aflatoxin adducts in serum of Gambian children was associated with lower salivary secretory IgA concentrations (Turner *et al.*, 2003). In animals, AFB_1-induced suppression of cell mediated immunity has been demonstrated using delayed type hypersensitivity in rats orally administered either 300 or 600 g/kg bw of AFB_1 sub-chronically (Raisuddin *et al.*, 1993). The evidence in favor of the immunosuppressive effects of aflatoxins highlights the broad nature of the potential effects of these toxins on human health.

1.1.5 The role of AFB_1 exposure in growth impairment among children

In children between 0 and 59 months, child growth can be evaluated using four WHO indicators namely; height or length for age, weight for age, weight for height, and more recently BMI for age Z scores (WHO Multicentre Growth Reference Study Group, 2006). Epidemiological studies have shown that aflatoxin exposure is associated with stunting, a condition where a child's height for age is below 2 standard deviations from the median of the WHO child growth standard. Aflatoxin related growth impairment has been reviewed and reported in several publications (Khlangwiset *et al.*, 2011; Smith *et al.*, 2015; Turner, 2013). From a public health point of view, stunting is associated with poor mental development which predisposes children to high risks of long-term cognitive impairment, susceptibility to infections, and nutrition related chronic diseases later in life (Turner, 2013; Wu *et al.*, 2014b). A longitudinal study among 16-37 months old Beninese children revealed a strong negative correlation between AFB_1 albumin adducts and height increase over an eight months period. Furthermore, the study reported an average 1.7 cm decrease in height of children among the highest quartile of AFB_1-albumin concentration relative to the lowest quartile (Gong *et al.*, 2004). Growth suppression may also occur due to prenatal exposure and is associated with reduced birth weight and length (Supriya *et al.*, 2016; Turner *et al.*, 2007). Although not fully elucidated, the mechanism by which AFB_1 affects child growth is thought to be via its binding to RNA thus inhibiting protein synthesis. Alternatively, growth impairment may be caused indirectly by immunosuppression which increases susceptibility of the AFB_1-exposed children to enteric infections, leading to diseases such as diarrhea (Turner, 2013). The weaning stage has been identified as one of the critical stages during which the slowest aflatoxin-induced growth faltering occurs (Turner, 2013; Wild

and Gong, 2010). Therefore, exposure to AFB_1 needs to be monitored and appropriate measures taken to prevent its associated long-term effects.

1.1.6 Role of AFB_1 in hepatocellular carcinoma

A recent global cancer statistics report estimated that, there were about 780,000 new HCC cases annually (Torre *et al.*, 2015). Of these, 25% are attributed to chronic exposure to aflatoxins thus making these toxins a major risk factor in HCC (Liu and Wu, 2010). It is worth noting that, out of the four naturally occurring aflatoxins, only AFB_1 has been implicated in HCC. This is due to the presence of a double bond between the Carbon-8 (C8) and C9 of AFB_1 which allows for easy epoxidation to form a highly reactive electrophilic intermediate ─ the AFB_1-8,9-exo epoxide. The mechanism of AFB_1-induced carcinogenicity involves binding of the AFB_1 epoxide to guanine bases of DNA to form the AFB_1-N^7- guanine adducts (Bedard and Massey, 2006). The DNA adduct formed may undergo ring opening reactions to form AFB_1-formamidopyrimidine (AFB_1-FAPY), which is a stable metabolite. Alternatively, subsequent depurination of the DNA adduct occurs and results in formation of an apurinic site (Smela *et al.*, 2001). These sites tend to be re-occupied by adenine that naturally binds to thymine hence inducing a specific arginine to serine mutation of the p53 tumor suppressor gene via a guanine (G): cytosine (C) to adenine (A): thymine (T) transversion at codon 249 (Bedard and Massey, 2006; Turner, 2013). The mutation inhibits apoptosis and production of a p53 transcription factor that plays a key role in cell cycle regulation thereby promoting cell proliferation (Zilfou and Lowe, 2009). Accumulation of adducts coupled with new mutations as a result of long-term exposure to AFB_1 is believed to cause progression to cancer. Therefore, given the overwhelming evidence from both animal and epidemiological studies linking aflatoxin exposure to HCC (Kew, 2013; Liu and Wu, 2010; Wu and Santella, 2012), IARC classified naturally occurring mixes of aflatoxins as Group 1 human

carcinogens (Liu and Wu, 2010; Wild and Turner, 2002). The risk of HCC is multiplicatively increased by hepatitis B viral (HBV) infections (Groopman *et al.*, 2008). Previous studies have reported that the risk of hepatocellular carcinoma among people who are chronically exposed to both AFB_1 and HBV infection is about thirty times greater than in individuals who are exposed to only AFB_1 (Liu and Wu, 2010; Williams *et al.*, 2004).

1.2 Trichothecenes

Trichothecenes are a family of more than 200 fungal toxins produced predominantly by *Fusarium* species. Other genera of fungi including *Trichothecium, Trichoderma, Myrothecium, Verticimonosporium, Cephalosporium*, and *Stachybotrys* are capable of producing trichothecenes. Fusaria are common plant pathogens and cause wilts, blights and ear rots in corn, wheat, barley, and oats (McCormick *et al.*, 2011). Trichothecene mycotoxins are esters of sesquiterpenoid alcohols consisting of a trichothecene tricyclic ring with a double bond at the C9-C10 and an epoxide at C12-C13 positions (Fig. 2). Due to their small molecular weight and amphipathic nature, trichothecenes are easily absorbed across the gastrointestinal membranes and are quickly distributed to various body organs and tissues. Amphipathic molecules have both hydrophilic and hydrophobic parts. The rank order for the distribution of trichothecenes in organs and tissues of mammals is; Kidney > heart > plasma > liver > thymus > spleen > brain (Pestka and Smolinski, 2005). The sensitivity of animals to trichothecenes also varies, with pigs as the most sensitive, followed in order by rodents, poultry, and ruminants as the least sensitive (Pinton *et al.*, 2012a). The higher tolerance in ruminants as compared to monogastric animals is attributed to detoxification by rumen microflora prior to absorption. Thus, because of the similarity between the human and the pig gastrointestinal systems, it is reasonable to suggest that

humans are as sensitive as pigs to toxic effects of trichothecenes (Pestka and Smolinski, 2005; Wu *et al.*, 2014b).

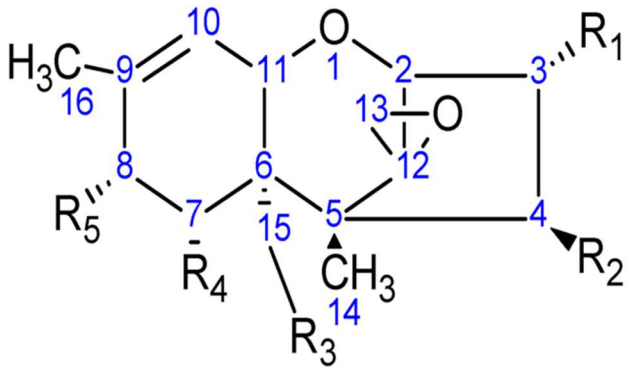


Figure 2. General structure of trichothecenes (Shank *et al.*, 2011)

Trichothecenes can be classified as type-A, -B, -C, or -D depending on the substitution on the 12,13-epoxytrichothethec-9-ene ring system. Of these, simple type-A and type-B are the most common toxins encountered in foods. Type-A trichothecenes have a carboxyl group at C8 and they include diacetoxyscirpenol, neosolaniol, T-2 and HT-2 toxins. They are produced predominantly by soil fungi *F. sporotrichoides*, *F. poae*, and *F. acuminatum*, and are the most toxic of the trichothecenes (McCormick *et al.*, 2011). Type-B trichothecenes have a keto function at C8 and they include deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, nivalenol, and 4-acetylnivalenol (also known as fusarenon-X). They are less toxic, but more prevalent in food than type-A trichothecenes. Among the type-As, T-2 is the most studied while overall, DON is the most researched trichothecene. Although, most studies have been conducted using animal models (Wu *et al.*, 2014b).

Trichothecenes contaminate food crops in all regions of the world. However, the terrestrial distribution of trichothecene-producing *Fusarium* species as well as the type of toxins produced tends to vary among regions. For example, In North America the *Fusarium spp* predominantly

produces DON while in Japan the dominant species are those that produce both DON and nivalenol (Pestka, 2010b; van der Lee *et al.*, 2015). These toxins are very resistant to milling (Pestka, 2010b) and other thermal processes including extrusion (at temperature as high as 150˚C) (Bullerman and Bianchini, 2007), frying (> 200˚C), and steaming (185˚C; 6 min) (Kabak, 2009) hence, they remain present in cereal products.

1.2.1 DON, 3-ADON, and 15-ADON

Deoxynivalenol or vomitoxin is the most prevalent and most studied trichothecene. It is produced primarily by *F. graminearum* and *F. culmorum*. In addition, these fungi molds tend to also produce small quantities of 3-ADON and 15-ADON which are precursors of DON. The foods commonly contaminated include maize, barley, oats, rice, and rye. Ingested DON is rapidly absorbed and distributed, reaching maximum concentrations in various tissues after 15-30 min. In the human liver, glucoronidation is the major detoxification biotransformation of DON to DON glucuronide which is ultimately excreted in urine. In mice, DON may also be reduced to de-epoxy DON which is non-toxic (Pestka, 2010b). Acute exposure to DON causes abdominal pain, diarrhea, vomiting, anorexia and fever. Moreover, extremely high doses (> 27 mg/kg bw/day) can be fatal in pigs (Pestka and Smolinski, 2005). Previous studies have shown that sub-acute exposure of pigs to as low as 0.8-6.0 mg/kg of DON causes inflammation of the intestinal mucosa (Pinton *et al.*, 2012a). In animals, and potentially in humans, chromic exposure to low doses of DON is associated with decreased food intake, decreased weight gain, decreased growth, and impaired immune system function. Notably, decreased ability to fight infections including *Salmonella enteritidis* and *Listeria monocytogenes* (Pestka and Smolinski, 2005). As a result, several countries around the world have set regulatory limits for DON in both human and animal feed. In 2001, JECFA set a provisional maximum TDI for DON at 1 ppm based on

effects on growth, reproduction and immune system function in animals (Canady *et al.*, 2001a). Following a review of additional studies, JECFA concluded that the toxicities of 3-ADON and 15-ADON were equal to that of DON so, the PMTDI was extended to include 3-ADON and 15-ADON. In 2011, the committee also derived an acute reference dose (ARfD) of 8 µg/ kg bw/day for the three toxins using benchmark dose modeling of the emetic endpoint in pigs (JECFA, 2011). Similarly, the US-FDA issued a recommendation of 1 ppm for maximum levels of DON in finished wheat and other cereal products intended for human consumption (FDA, 2010).

1.2.2 Nivalenol and fusarenon-X

Nivalenol is an 8-keto trichothecene characterized by a hydroxyl group at C4. Acetylation of NIV results in substitution of the hydroxyl group with an acetyl to generate FX toxin and vice versa i.e. deacetylation of FX yield NIV. These toxins are mainly produced by *F. cerealis, F. poae, and F. nivale* and to a lesser extent *F. graminearum*. Contamination of cereal-based foods has been reported in all parts of the world, especially in wheat, barley, corn, oats, and rye and their processed products including bread, malt, and beer (Ibáñez-Vea *et al.*, 2012). The oral toxicity of NIV lower than that of FX since NIV is poorly and inefficiently absorbed from the GIT while FX is very rapidly and efficiently absorbed. Interestingly, once FX is absorbed into plasma, it is quickly converted back to NIV. Hence, NIV is the form in which FX exerts its toxicity (Male *et al.*, 2016). Approximately 80% of the ingested NIV is excreted in feces as de-epoxy NIV while the majority of FX is excreted in urine (Pestka, 2010b). Most previous studies on NIV and FX toxins have been in vitro and in animals especially pigs and rodents. There is still limited epidemiological data on NIV which makes its risk evaluation difficult. Nonetheless, some previous in vitro studies using human cell lines have shown that NIV modulates immune system function, inhibits protein synthesis and induces apoptosis (Berek *et al.*, 2001; Cundliffe

and Davies, 1977). Animal studies have shown that acute exposure causes emesis, food refusal, diarrhea, impaired intestinal function, and death at extremely high doses. Moreover, sub-chronic and chronic exposure to NIV is associated with reduced food intake, reduced weight gain, retarded growth, impaired immune system, erythropenia, and reproductive and development toxicity (Pestka, 2010b). Currently, there is no evidence to suggest that either NIV or FX is carcinogenic. In 2002, the European Commission's Scientific Committee on Food set a temporary TDI of 0.7 g/kg bw for NIV based on growth retardation and immunotoxicity as the most critical endpoints (SCF, 2002). The same limit was later adopted and included in EU regulation No. 1881/2006 (Commission, 2006).

1.2.3 T-2 and HT-2 toxins

These toxins are simple trichothecenes produced mainly by soil fungi including *F. sporotrichioides, F. poae, F. equiseti,* and *F. acuminatum*. T-2 and HT-2 toxins are characterized by an isovareryl ester functional group at C8 and a hydroxyl group at C3 of the trichothecene tricyclic ring system (Desjardins *et al.*, 2007). They are produced in foods when the water activity is very high, normally above 0.88 hence, they are prevalent in water damaged grains such as over wintered wheat and poorly stored grains that become damp or wet. They are common contaminants in wheat, barley, corn, oats, rice, soybeans, and beans grown in temperate regions of the world. Acute exposure to sufficient doses of either T-2 or HT-2 causes mouth lesions, gastric lesions, emesis, feed refusal, bloody diarrhea, immunotoxicity, and may cause death. Prolonged exposure is associated with reduced weight gain, growth suppression, and severe gastrointestinal lesions. However, there is no sufficient evidence to suggest that these toxins cause cancer (Canady *et al.*, 2001b). Ingestion of T-2 and HT-2 is associated with alimentary toxic aleukia (ATA) in humans, a hemorrhagic disease characterized by mouth and

nose bleeding, vomiting, diarrhea, abdominal pain, and fever. Incidences of ATA in parts of Orenburg, USSR (modern day Russia) were linked to consumption of overwintered wheat (Bennett and Klich, 2003; Wu *et al.*, 2014b). Chronic exposure to T-2 is also associated with Kashin Beck Disease (KDB), a chronic degenerative osteoarthritic condition that causes short stature and may cause disability in adults. The symptoms of KDB have been subsequently verified using studies where animals have been sub-chronically exposed to T-2 toxin (Kang *et al.*, 2013). As a result of extensive evaluation of available literature on T-2 and HT-2 toxins, JECFA set a group PMTDI of 60 ng/kg bw per day for both T-2 and HT-2 based on altered leucocytes and erythrocyte counts in pigs (Canady *et al.*, 2001b).

1.2.4 Mechanism of toxicity of trichothecenes

As previously discussed, DON is the most prevalent and most studied trichothecene mycotoxin. Hence the mode of action discussed here is largely based on in vitro and in vivo studies involving DON. The mechanism of toxicity of trichothecenes involves their binding to DNA, cellular proteins, and protein-like molecules ultimately causing pathology (Wu *et al.*, 2013d). Structurally, the presence of an epoxide group confers toxicity to trichothecenes. The epoxide functional group is highly reactive to the nucleophilic sulfur atoms that are usually present as thiol, thioesters, and hydrogen sulfide functional groups in amino acids e.g. cysteine and methionine (Jarrett, 2015). In particular, trichothecenes bind to ribosomes of eukaryotic cells, inactivating the ribosome cycle consequently inhibiting initiation and chain termination. Thus, these toxins are potent inhibitors of DNA and protein synthesis in eukaryotic cells (Pestka, 2010d; Ueno, 1977). In addition, trichothecenes also interfere with translation by competitively inhibiting peptidyl transferase ─ an enzyme involved in initiation and elongation during protein synthesis (Pestka, 2010b). Some of the downstream pathophysiologic effects include aberrant

endocrine signaling, impaired growth hormone production, and disruption of translocation of nutrients and other materials in and out of the cell (Pestka, 2010d). Abnormal endocrine signaling following exposure to trichothecenes was recently shown in a mink model where the plasma concentration of PYY_{3-36} ─ a satiety hormone and 5-hydroxytryptamine, a neurotransmitter were significantly elevated (Wu *et al.*, 2013e; Wu *et al.*, 2016). Exposure to trichothecenes also alters intercellular communication, by activating mitogen activated protein kinases (MAPK) which promotes cytokines production and induces generation of reactive oxygen species, subsequently inducing programmed cell death (Pestka and Smolinski, 2005).

1.3 Fumonisins

Fumonisins are produced by *F. verticillioides, F. proliferatum, A. niger,* and other *Fusarium* species. They are common contaminants of maize, sorghum, beans, soy beans, and wheat in both hot climatic regions (Kumi *et al.*, 2014) and cool temperate regions (Bowers and Munkvold, 2014). There are more than ten fumonisin and fumonisin-like compounds that have been isolated and characterized. These include fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), fumonisin B_4 (FB_4) fumonisin B_6 (FB_6) and fumonisin BX (FBX) series (Scott, 2012). Of these, three types of fumonisins namely; FB_1, FB_2, and FB_3 are produced in nature, of which FB_1 is the most prevalent and most toxic (FDA, 2001). They are heat stable and non-volatile under ethanol processing conditions. Thus, they are prevalent in wine, beer, and other alcoholic products (Bowers and Munkvold, 2014). Fumonisins covalently bind to proteins and sugars during heat processing. Although these bound toxins remain bioavailable, they could be released in the gastrointestinal tract and subsequently absorbed in their free form (Scott, 2012). Once absorbed, fumonisins are rapidly distributed to the liver and kidneys where they inhibit ceramide synthase activity, ultimately interfering with sphingolipid biosynthesis (Ahangarkani *et al.*, 2014). In pigs,

exposure to fumonisins is associated with pulmonary edema, cardiovascular, and liver damage. Fumonisin B_1 modulates immune system function; chronic exposure is associated with decreased lymphocyte response to infection resulting in increased intestinal colonization by pathogens (Burel *et al.*, 2013). Acute exposure to FB_1 and FB_2 has been implicated in equine leucoencephalomalacia, a disease characterized by blindness, hyper-excitability, uncoordinated locomotion and death in horses (Giannitti *et al.*, 2011). In humans, growth faltering has been reported among children exposed to greater than 2 g/kg bw fumonisin per day (Turner, 2013). Epidemiological studies also indicate that exposure is associated with an increased risk of esophageal cancer and neural tube defects in humans (Wu *et al.*, 2014b). Fumonisins are classified by IARC as group 2B ─ possibly carcinogenic to humans (IARC, 2002a). As a result, several nations have set regulatory limits for fumonisins in food products. The European Union has set maximum limits in baby foods, breakfast cereals, and unprocessed maize at 0.2 ppm, 0.8 ppm, and 4 ppm, respectively (Scott, 2012) while the US-FDA recommended ranges for levels of total fumonisins ($FB_1 + FB_2 + FB_3$) in human food and animal feed are 2-4 ppm and 5-100 ppm, respectively (FDA, 2001).

1.4 Ochratoxins

Ochratoxins are crystalline polyketide-derived secondary metabolites containing a dihydro-isocoumarin moiety coupled to a phenylalanine group (Wang *et al.*, 2016). They are putatively produced by members of the *Aspergillus* and *Penicillium* species especially *A. ochraceus*, *A. niger*, and *P. verrucosum*. The naturally occurring ochratoxins co-produced in food include ochratoxin A (OTA), ochratoxin B (OTB), and ochratoxin C (OTC). Of these, OTA is the most toxic and most prevalent in food (Reddy and Bhoola, 2010). These toxins contaminate maize, wheat, barley, oats, beans, coffee, meat, and dairy products worldwide. Ochratoxins are very heat

stable (melting point =169˚C) thus, not completely eliminated from food during cooking or heat processing (Kőszegi and Poór, 2016; Wang *et al.*, 2016). In humans, more than 90% of the ingested OTA is absorbed into the blood stream where is strongly covalently binds to albumin. OTA is metabolized by in the liver and kidneys to either less toxic ochratoxin α, 10-hydroochratoxin A, and OTB, or a highly toxic lactone ring opened OTA (Kőszegi and Poór, 2016). While animals rapidly metabolize and excrete OTA, its half-life in humans is unexpectedly very long due to enterohepatic recirculation, protein binding, as well as reabsorption in the kidneys, which increases the internal dose and subsequently causing renal failure (Reddy and Bhoola, 2010). Most OTA is eliminated from the body via biliary excretion. Acute exposure is immunotoxic, hepatotoxic, and neurotoxic. Chronic exposure has been implicated in endemic nephropathy, a porcine and human kidney disease characterized by atrophy of proximal tubules, fibrosis and impaired tubular function (Castegnaro *et al.*, 2006; Malir *et al.*, 2016; Wang *et al.*, 2016). However, some studies have cast doubts over the nephrotoxicity of ochratoxins following observations that exposures in endemic regions were lower than the regulatory limits (Reddy and Bhoola, 2010). Instead, some scholars have suggested possible nephro-pathogenicity via interactions with other environmental contaminants such as aristolochic acid, a phytochemical produced by aristolochiaceae plants and citrinin, a polyketide mycotoxin produced by *Penicillium* and *Aspergillus spp* and shown to activate oxidative stress responses (Pascual-Ahuir *et al.*, 2014; Pfohl-Leszkowicz, 2009). Based largely on data from animal studies, the IARC classified ochratoxins as Group 2B since they may potentially cause cancer in humans (Kőszegi and Poór, 2016). Therefore, ochratoxins are among the strictly regulated mycotoxins. The FAO/WHO joint expert committee on food additives (JECFA) issued a provisional TWI of 112 ng/kg bw/week (JECFA, 2001a). The European

scientific committee on food set an even more conservative limit of 5 ng/kg bw/day (Reddy and Bhoola, 2010) but, thus far, there is no regulatory limit for OTA in the United States (Cappozzo *et al.*, 2017).

1.5 Zearalenone

Zearalenone (ZEA) is a macrocyclic, nonsteroidal mycotoxin, produced by *F. roseum, F. culmorum* and *F. graminearum* (Hueza *et al.*, 2014). It has been reported in cereals from all parts of the world. ZEA is commonly detected in maize, wheat, barley, oats, sorghum and rice. ZEA can also be secreted in milk when cattle consume greater than 12 mg/kgbw/day of the toxin (Zinedine *et al.*, 2007). The stability of ZEA during storage, milling and heat processing of cereals (Hueza *et al.*, 2014) mean the toxin also appears in their processed products such as baked bread, fermented products such as beer and in processed animal feed. ZEA is rapidly absorbed by intestinal cells (Pfeiffer *et al.*, 2011) where it is subsequently transformed by 3α- and 3β-hydroxysteroid dehydrogenases to predominantly a more estrogenic active isomer α-zearalenol and a slightly less potent β-zearalenol (Keller *et al.*, 2015). The isomers are distributed to several targets which include the liver, uterus, ovaries and testes. ZEA and its reduction isomers mimic the structure of estrogen and thus binds with the estrogen receptors consequently altering the function of the endocrine system and causing undesirable effects (Li *et al.*, 2012). Common reproductive effects in animals include; enlargement of reproductive tracts in pigs, alteration of ovulation, fibrosis of the uterine walls, and fetal development abnormalities (Keller *et al.*, 2015). A hypothetical model using mitoproteomics showed that ZEA exerted reproductive toxicity by increasing fatty acid uptake, altering lipid metabolism and decreasing hormone release (Li *et al.*, 2014). Studies have also demonstrated that ZEA is immunotoxic since endocrine receptors are expressed by immune cells such as monocytes and NK cells (Hueza *et*

al., 2014). Therefore, based on its estrogenic activity in the pig model, the European Food Safety Authority (EFSA) established a tolerable daily intake (TDI) of 0.25 μg/kg bw/day for Zearalenone in food (EFSA, 2011; Karlovsky *et al.*, 2016).

2. Challenges facing global mycotoxin regulatory efforts

Mycotoxin safety regulation is both a public health and an economic issue to society and national governments (Cardwell *et al.*, 2001). It is reasonable that, in the end, a country adopts regulatory standards that its producers and consumers can afford without excessively limiting access to valuable food commodities, which may occur as a result of over regulation. Therefore, policy makers go through rigorous cost-benefit analyses to determine the most feasible means to reduce mycotoxin exposure. However, in some instances, compromises are made that disregard or fail to utilize sound scientific data in favor of economic practicability. As a result, from a public health perspective, current mycotoxin regulations may not actually be sufficiently protective. Here, we discuss some of the key challenges encountered in regulating fungal toxins.

First, mycotoxins tend to co-occur in food and collectively cause an array of acute and chronic adverse effects (Ibáñez-Vea *et al.*, 2012; Smith *et al.*, 2016). This can be either a consequence of contamination with fungal species such as *F. poae* and *F. graminearum* that are capable of producing several toxins or co-contamination of food by a variety of fungal species. *Fusarium poae* for instance, is capable of synthesizing fusarin, culmorin, NIV, FX, T-2, HT-2, neosolaniol, monoacetoxyscirpenol, and diacetoxyscirpenol (Dinolfo and Stenglein, 2014; Stenglein, 2009). Co-contamination of staples by multiple fungi has also been previously reported (Greco *et al.*, 2015; Njobeh *et al.*, 2009). The total toxicity of such foods is not normally ascertained and even when the prevalence of individual toxins is determined, their interaction is often ignored. This is

because toxicological investigations are carried out single molecule studies, allowing the evaluation of potencies of toxins as though they were stand-alone compounds, without any interference from both food matrices and other metabolites. More recently studies on dose-activity relationships involving combined mycotoxins have revealed interesting cooperative effects including synergistic and antagonistic effects which unfortunately are not considered in the current mycotoxin regulations (Alassane-Kpembi *et al.*, 2014; Yang *et al.*, 2017). Irrespective of the sources of mycotoxin cocktails, current regulations are not built to assess risks associated with mycotoxins in mixtures. Secondly, in relation to the number of known toxic fungal metabolites, currently available mycotoxin regulations are very narrow in scope. Even the so called comprehensive mycotoxins regulations such as those in the EU (EC No. 1881/2006) represent a very small proportion of the entirety of mycotoxins that potentially contaminate food. Over the years scientists have made a commendable effort to identify more mycotoxins and their fate in vivo, and indeed hundreds of compounds have been discovered. For example, there are more than 200 trichothecenes that have been identified so far (McCormick *et al.*, 2011) but only a handful of type-A and type-B trichothecenes – in particular, DON, NIV, and T-2 toxin have had a significantly high number of toxicological studies conducted on them. Even then, their mechanisms of action are not fully understood. Moreover, mycotoxins can be found in a diverse range of foods, far beyond what current regulations cater for. The mere fact that there are still so many mycotoxins whose methods of detection are not yet fully developed, and whose distribution both geographically and in foods is not known, means that it might take many years before their regulations are developed. Finally, due to differences in resources as well as perceived risk, different nations around the world have set different regulatory limits for certain mycotoxins in food. As a result, wealthy nations with similar regulatory standards trade among

each other whereas the majority of low developed countries are restricted to local trade. Such scenarios further compound the health burden of mycotoxins on communities - the mycotoxins are not distributed via international trade but instead, the exposure is concentrated within small geographical zones (Cardwell *et al.*, 2001; Wu and Guclu, 2012).

CHAPTER TWO: Serum level of AFB_1-lysine adducts in Ugandan children aged 1-5 years

The data in this chapter will be published in Male, D., Mitchell, N.J., Linz, J.E., Pestka, J.J., Egner, P.A., Groopman, J.D., Nakimbugwe, D., Kaaya, A.N., and Wu, F.

Abstract

Aflatoxins are a highly toxic group of naturally occurring mycotoxins produced by *Aspergillus parasiticus* and *A. flavus* molds. Aflatoxins commonly contaminate staple foods including maize, peanuts, cottonseed, sunflower seeds, and tree nuts; primarily in tropical and subtropical regions of the world. In humans, aflatoxin exposure has also been linked to immune system dysfunction, child growth impairment, and acute toxicity and death at high doses. Despite widespread food contamination with aflatoxins in Uganda, the health burden among children remains unknown due to ineffective regulations and limited testing capacity. In this cross-sectional study, we sampled blood from 46 children (26 male and 20 female), aged 12-59 months, from different regions of Uganda. The Serum concentration of AFB_1-lysine adducts was determined by isotope dilution mass spectrometry. All samples had detectable concentrations of AFB_1-lysine adducts (mean, 16.3 pg/mL; range, 1.4 - 136.6 pg/mL). We observed differences in aflatoxin exposure among regions ($p = 0.0114$), but there were no significant differences in exposure by age group, sex, and residence type. Levels of aflatoxin exposure among Ugandan children are consistent with concentrations associated with growth impairment in other epidemiological studies.

Keywords: *Aspergillus*, aflatoxin, biomarkers, immune dysfunction, child dietary exposure

1. Introduction

Aflatoxins are a highly toxic group of naturally occurring compounds produced as secondary metabolites of polyketide synthase-A activity in *Aspergillus* molds. Aflatoxin B_1 is the most frequently occurring and most toxic among the naturally occurring aflatoxins (Wild and Turner, 2002). Food crops susceptible to aflatoxin contamination include maize, peanuts, cottonseed, sun flower, pecans, pistachios, and dried fruit. The incidence of aflatoxins in food is higher in tropical regions of the world, where high temperatures and humidity favor *Aspergillus* growth. Humans are primarily exposed to aflatoxins when they ingest contaminated food. In many parts of sub-Saharan Africa, the level of exposure among children significantly increases at weaning when aflatoxin-contaminated solid cereal foods are introduced (Gong *et al.*, 2003; Obade *et al.*, 2015).

Since the 1960s, it has been known that dietary aflatoxin exposure causes hepatocellular carcinoma (liver cancer) in humans. Moreover, chronic co-exposure to aflatoxins and hepatitis B infection synergistically increase the risk of liver cancer (Groopman *et al.*, 2008; Liu *et al.*, 2012; Wu *et al.*, 2014b). The International Agency for Research on Cancer (IARC) classifies naturally occurring mixtures of aflatoxins as a Group 1 carcinogen (IARC, 2002c). Chronic exposure to aflatoxins in humans is associated with child growth impairment and immune system dysfunction, which can make individuals more susceptible to infectious disease (Gong *et al.*, 2002; Gong *et al.*, 2004; Khlangwiset *et al.*, 2011; Qian *et al.*, 2014). Most developed countries have set regulatory limits for aflatoxin levels in food between 4 ng/g and 40 ng/g (Wu and Guclu, 2012). Unfortunately, in many low income sub-Saharan African countries where large amounts of potentially contaminated maize and peanuts are consumed, these regulatory standards

are either absent or not adequately enforced (Wu *et al.*, 2013a). Additionally, these countries lack the diagnostic capacity to determine levels of Aflatoxin exposure (Lingwood *et al.*, 2008).

Liu and Wu (2010) estimated aflatoxin exposure in adults in multiple nations and regions worldwide, through maize and peanut consumption (Liu and Wu, 2010). Prenatal exposure to aflatoxins has also been reported (Hernandez-Vargas *et al.*, 2015; Supriya *et al.*, 2016; Wild and Turner, 2002). Animal studies have shown that exposure during pregnancy causes reduced birth weight as well as irregular estrus and suppressed fertility of the progeny during adulthood (Supriya *et al.*, 2016). In humans, exposure to aflatoxins during the first trimester increases DNA methylation in white blood cells which is associated with an increased risk of stunting and hepatocellular carcinoma later in life (Hernandez-Vargas *et al.*, 2015). Breast feeding children are at a high risk of ingesting AFM_1 a metabolite of AFB_1 which itself is toxic - in breastmilk (Diaz and Sánchez, 2015; Fallah *et al.*, 2015; Garrido *et al.*, 2003).

Despite the widespread contamination of foodstuffs with aflatoxins in sub-Saharan Africa, there is limited data on the levels of these harmful toxins in blood of children in Uganda. This limitation of data makes it very difficult to assess the impact of aflatoxins on immune system function, child growth, and risk of hepatocellular carcinoma (later in life) among Ugandan children. In this survey, we determined the concentration of AFB_1-lysine adduct, a well-established biomarker of chronic aflatoxin exposure (Scholl and Groopman, 2008; Scholl *et al.*, 2006), in samples of serum collected from different regions of Uganda in order to estimate the aflatoxin burden in children between 1 and 5 years of age.

2. Methods

2.1 Study population

This cross sectional study was conducted within the context of a larger research project to evaluate the relationship between aflatoxin B_1 exposure and the severity of malaria among Ugandan children aged between 1 and 5 years. The participants were screened and recruited from children presenting with symptoms of malaria at hospitals or health clinics. The study sites included Okidi Health Center III, Kitgum Regional Referral Hospital, Pajule Health Centre IV, and Lalogi Health Center IV in northern region, Iganga Regional Referral Hospital, Iganga Health Center III in eastern region, and Mbarara Holy Innocents Children's Hospital, Kinoni Health Center IV, and Bwizibwera Health Center IV in western Uganda. The training for research assistants, patient screening, and sample collection were done between December, 2015 and January, 2016. The study protocols were reviewed and approved by Michigan State University Biomedical and Health IRB (MSU-BIRB), Makerere University School of Health Sciences-IRB (MakSHS-IRB) and the Uganda National Council for Science and Technology (UNCST). Informed written consent was obtained from the parent or legal guardian of each participant.

2.2 Participant screening and recruitment

Screening was done by a well-trained nurse, recruited at the host hospital, using a questionnaire (See Appendix). We endeavored to include a relatively equal number of male and female participants between the ages of 1 and 5 years. The parents or legal guardians of the children who were eligible to participate in the study were requested to speak with the researchers and subsequently could choose to provide written consent for participation. The process took place in a private and non-coercive environment provided by the health center. The screening consent

forms and the research participation consent forms were translated into selected local languages commonly spoken in Uganda including Luganda, Runyankole, Acholi, and Langi. The children that participated in the study received malaria treatment before leaving the hospital. In cases where treatment was not available at the hospital, the research personnel provided standard malaria treatment to the participant.

2.3 Blood sample collection and handling

For each child, 5 mL of blood was collected by the hospital laboratory technician. The samples were kept at ambient temperature for 30-60 mins to allow separation of serum from cells and clotting factors. Aliquots of the serum were transferred to 2 mL cryovials in duplicate and temporarily kept in the freezer at the hospital. They were then transported in cool boxes filled with ice packs, from the hospitals to Makerere University where they were stored at -80˚C. The samples were then shipped in one batch, on dry ice to Johns Hopkins University, USA, for analysis of AFB_1-lysine adducts.

2.4 Quantitation of AFB_1-lysine adducts in serum samples

All serum samples from the Ugandan children were analyzed using the isotope dilution mass spectrometry method previously described by Groopman and colleagues (Groopman *et al.*, 2014; McCoy *et al.*, 2005). Briefly, about 200 µL of the sample was mixed with an internal standard (10 µL × 0.1 ng AFB1-D4-lysine/mL) and pronase solution (250 µL, 13 mg/mL PBS), then incubated for 18 hours at 37 °C. The prepared serum sample-standard mixture was analyzed by ultra-performance liquid chromatography (UPLC)-tandem mass spectrometry. In this technique, the internal standard parent molecular ion [(M + H) +, m/z 461.3] is fragmented to yield an ion at m/z 398.2. The AFB_1-lysine molecular ion (m/z 457.2) is fragmented to yield an ion at m/z 394.1. A 10-point isotopic dilution standard curve was generated by triplicate injection of AFB_1-

D4-lysine standard (100 pg) mixed with varying amounts of AFB_1-lysine prepared via 2-fold serial dilutions. The data was fitted using the least squares method with a 1/x weighting factor (x= varying amounts of 0-0.4 ng AFB1-lysine). The limit of quantitation for this analysis is 0.5 pg AFB_1-lysine per milligram of albumin (McCoy *et al.*, 2005).

2.5 Statistical analysis

We conducted univariate analysis to test for normality, determine central tendency, and dispersion in the data. In addition, we calculated descriptive statistics for the concentration of AFB_1-lysine adducts in the serum samples. The participants' aflatoxin concentration results were categorized by region (Eastern, Northern, and Western), gender (male and female), residential status of household (urban, peri-urban, and rural) and age group (12-23, 24-35, 36-47, and 48-59 months). The differences in mean AFB_1-lysine biomarkers in sera of the children from different regions, residence type, and age groups were evaluated using the Kruskal-Wallis (KW) test (McDonald, 2009). In instances where the KW test indicated a statically significant difference, multiple pairwise posthoc comparisons were performed with a Bonferroni type-1 error correction to analyze differences between the individual sample pairs. The Mann-Whitney-Wilcoxon test was used to evaluate the difference in mean AFB_1-lysine adduct concentration between male and female participants. The level of significance, α was set at $p < 0.05$. In post hoc analysis, the null hypothesis was rejected if the p-value was less than the adjusted level of significance. All analyses were performed using SAS University Edition (SAS Corporation, Cary, NC, USA).

3. Results

We tested sera from 46 children aged between 12 and 59 months including 26 male and 20 females from three regions (Eastern, Northern, and Western) of Uganda. All samples had detectable concentrations of AFB_1-lysine adducts which ranged from 1.42 to 136.64 pg/mg albumin, with a mean and median concentration equal to 16.3 pg/mg and 10.9 pg/mg albumin, respectively. The level of AFB_1 was not normally distributed (Shapiro-Wilk test, W = 0.576, Pr < W = 0.0001). The mean concentration of AFB_1-lysine biomarkers in sera of the different categories of children were summarized (Table 1). The Kruskal-Wallis global test indicated that there were significant statistical differences among the concentrations of AFB_1-lysine adducts in sera of children taken from different regions of Uganda (KW chi square test statistic = 8.95, Pr > chi square = 0.0114).

The level of exposure to AFB_1 was highest among children in Eastern Uganda, followed by those from the Northern region while the Western Uganda children had the lowest concentrations (Fig. 3). Multiple post hoc pairwise comparisons conducted using the Mann-Whitney U test, with Bonferroni correction, revealed that the concentration of AFB_1-lysine adducts was significantly higher in sera of children from Eastern Uganda than that taken from children living in the Western region (2-Sided Pr > |Z| = 0.011) (Table 2). In addition, the post hoc analysis indicated that there were no significant differences between the levels of AFB_1 biomarkers in sera of children from Northern and Eastern or from Northern and Western regions (2-Sided Pr > |Z| = 0.113 and 0.068, respectively).

Table 1. Comparison of the levels of AFB_1-lysine adducts among regions, age-groups, residence types, and gender in serum of Ugandan children aged 1-5 years

Groups	Sub-groups	AFB_1 adducts (pg/mg)	p-value
Region	Eastern (14)	20.9 ± 3.5	0.0114
	Northern (20)	18.4 ± 6.6	
	Western (12)	7.4 ± 1.9	
Age group	12-23 months (12)	17.4 ± 4.3	0.7617
	24-35 months (10)	22.0 ±12.9	
	36-47 months (10)	15.5 ± 3.2	
	48-59 months (14)	11.9 ± 2.7	
Residence	Urban (04)	18.5 ± 4.3	0.4280
	Peri-urban (07)	15.0 ± 6.7	
	Rural (35)	16.3 ± 3.9	
Sex	Male (26)	13.5 ± 2.1	0.8342
	Female (20)	20.0 ± 6.7	

Number of participants in each sub-group is given in parenthesis. Concentrations recorded as means ± S.E. Level of significance, $\alpha = 0.05$. n = 46.

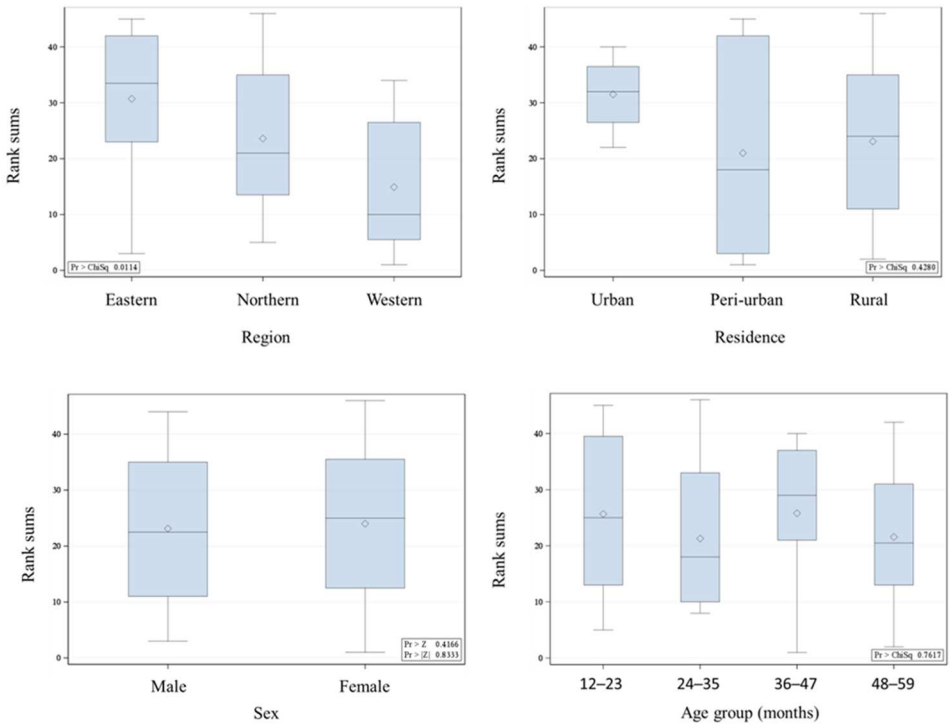


Figure 3. The distribution of Wilcoxon scores for the concentration of aflatoxin B_1-lysine adducts in serum of Ugandan children aged 1-5 years

The Mann-Whitney U test results indicated that there were no significant differences between the concentration of AFB_1 biomarkers in male and female children ($Pr > |Z| = 0.834$). Thus, the level of exposure to aflatoxins was not dependent on the gender of children. Likewise, statistical analyses did not reveal sufficient evidence in our data to indicate that the concentration of AFB_1-lysine adducts varied with age or residence type (KW test statistic = 1.16 and 1.70, Pr > Chi square = 0.762 and 0.428, respectively) (Table 1). Our results thus show that the level of exposure to aflatoxins is similar among children between 1 and 5 years and does not vary with household residence type.

Table 2. Multiple pairwise post-hoc analysis for mean AFB_1-lysine adducts concentrations in serum of children aged 1-5 years taken from different regions of Uganda

Group pairs	Mean difference [1] (pg/mL)	MWW [2] test statistic	p-value [3] (2-tailed)
Eastern-Western	13.53 ± 5.423	108	0.011
Eastern-Northern	2.56 ± 10.071	292	0.113
Northern-Western	10.97 ± 8.484	149	0.068

Mean difference recorded as mean ± S.E. MWW = Mann-Whitney-Wilcoxon. Level of significance, $\alpha = 0.05$. Bonferroni corrected level of significance, $\alpha^* = \alpha/3$. The difference between pairs is significant when the 2-sided p-value is less than α^*

4. Discussion

The purpose of this study was to assess the level of aflatoxin exposure and to evaluate the effect of age, sex, region, and residence type on the distribution of aflatoxins among Ugandan children aged 1-5 years. To this end, we found detectable levels of AFB_1 biomarkers in all serum samples tested which suggests that, the exposure is universal among children in Uganda. The widespread contamination is promoted by the warm and humid climate, characteristic of Uganda, which is conducive for mold growth (Kaaya and Warren, 2005). Indeed mold contamination remains one of the biggest threats to food security in Uganda (Shively and Hao, 2012). Our results are consistent with a recent pilot study that reported detectable AFB_1-albumin adducts in all participants including exclusively breastfed Ugandan children (Asiki *et al.*, 2014).

Furthermore, we compared our results with previous epidemiological studies conducted in other regions of the world. However, most available studies have used different methods, including ELISA, HPLC, and IDMS, with varying sensitivity and specificity to determine the level of aflatoxin exposure, which makes inter-study comparisons difficult (McCoy *et al.*, 2008). Although data from these methods are highly correlated, the values measured using ELISA are on average 2.6-fold greater than those obtained using the IDMS method (Scholl *et al.*, 2006). This is because, the ELISA method measures other cross reactive AFB_1 adducts in addition to the AFB_1-lysine while, the IDMS method used in our study specifically measures AFB_1-lysine adducts. Therefore, to make meaningful comparisons with previous studies that used the ELISA, we multiplied the AFB_1-lysine values from our study by a factor of 2.6 to obtain a geometric mean of 42.4 pg/mg albumin with a range of 3.7-355.3 pg/mg of albumin. This transformation makes our results similar and consistent with previous seminal publications including Gong *et al.* (2002) (mean, 32.8 pg/mg; range, 5-1064 pg/mg) and Gong *et al.* (2004) (mean, 43 pg/mg; range, 9.2-148.1 pg/mg), in which immunosuppressive effects and growth impairment were reported (Gong *et al.*, 2002; Gong *et al.*, 2004).

In the study to evaluate the effect of aflatoxins on child growth among children in Benin, Gong and coworkers reported a 1.7 cm retardation in height increase between the highest and lowest aflatoxin exposed quartiles over a period of 8 months (Gong *et al.*, 2004). The levels of aflatoxin-albumin adducts that were associated with the growth suppression in this study among Benin children were comparable to those we found among Ugandan children. Similarly, an earlier study in Benin and Togo found a strong negative correlation between exposure to AFB_1 and stunting (HAZ, $p = 0.001$) (Gong *et al.*, 2002) suggesting that, these toxins indeed negatively affect child growth.

Conversely, a recent study on Nepalese children to investigate the relationship between AFB_1 exposure on child growth did not find any association between the concentrations of AFB_1-albumin adducts (mean, 9.4 pg/mg albumin; range, 1.4-387.4 pg/mg albumin) and the child growth metrics (length for age (LAZ), p = 0.98; weight for age (WAZ), p = 0.70; weight for length (WLZ), p = 0.69) at 15, 24, and 36 months of age (Mitchell *et al.*, 2017). Although the mean levels of biomarkers detected in the Gong *et al.* (2002), Gong *et al.* (2004), and Mitchell *et al.* (2017) studies were comparable to those for Ugandan children, their effect on child growth were contrasting. Therefore, more epidemiological studies are needed to generate additional data to accurately quantitate the relationship between aflatoxin exposure and growth impairment.

Several animal studies have shown that AFB_1 modulates immune system function (Jiang *et al.*, 2015; Larypoor *et al.*, 2013; Long *et al.*, 2016; Qian *et al.*, 2014). However, there are few epidemiological studies that have investigated aspects of AFB_1-induced immune responses including cytokine production and expression of various phenotypes by leucocytes (Jiang *et al.*, 2005; Jiang *et al.*, 2008; Turner *et al.*, 2003). For example, in a seminal study conducted on 6-9 year old Gambian children, Turner and coworkers found that, the mean concentration of secretory immunoglobulin A (sIgA) in saliva of children with detectable AFB_1 was lower than that measured in saliva of children with undetectable AFB_1 levels (detectable AFB_1, sIgA = 50.4 g/mg protein; undetectable AFB_1, sIgA = 70.2 μg/mg protein; $p < 0.0001$) (Turner *et al.*, 2003). In the Jiang et al., (2005) study, the subjects were categorized into either low or high exposure group if their plasma contained either lower or higher than 0.9 pmol AFB_1/mg albumin, respectively. Based on this criterion, the group with a higher exposure had lower percentages of lymphocytes expressing $CD3^+CD69^+$ and $CD19^+CD69^+$, as well as perforin and granzyme A expressing $CD8^+$ cells than the lower AFB_1 exposure group (Jiang *et al.*, 2005). Although the

mean levels of AFB_1 adducts shown in previous studies were generally higher than those determined in Ugandan children, the mere fact that detectable levels among Gambian children were associated with reduced IgA secretion suggests a similar risk among Ugandan children.

The risk of AFB_1 induced cancer, in particular HCC has been extensively studied using the aflatoxin-N^7-guanine adducts biomarker (Qian *et al.*, 1994; Wu *et al.*, 2007). In this study, the level of exposure was assessed using the AFB_1-lysine albumin adducts. Interestingly, studies have shown that the concentration of AFB_1-albumin adducts is negatively associated with global DNA methylation, suggesting an additional epigenetic pathway through which AFB_1 causes hepatocellular carcinoma HCC (Wu *et al.*, 2013b). Genome-wide DNA hypomethylation among infants may be due to prenatal exposure of their mothers to AFB_1 (Hernandez-Vargas *et al.*, 2015), who then expose their children via umbilical cord blood (Abdulrazzaq *et al.*, 2002; Hsieh and Hsieh, 1993; Partanen *et al.*, 2009). Despite the available evidence linking AFB_1 exposure to DNA methylation, it is not clear what the lowest chronic AFB_1 levels (or doses) are that cause a significantly increased risk of HCC later in life. Hence the relationship needs to be explored further to ascertain the dose-response relationship.

Our study revealed regional differences in levels of aflatoxin exposure, and the rank order for the concentration of AFB_1 adducts was: Eastern > Northern > Western region. The results indicated that children from Eastern Uganda were exposed to significantly higher levels of aflatoxins than their Western Uganda counterparts. This finding is consistent with a previous study which showed that the incidence and concentration of aflatoxin in maize vended in Uganda varied across ecological zones (Kaaya and Kyamuhangire, 2006). The study reported that moist mid-altitudes (900–1500m) had the highest contamination followed by the dry mid-altitude (900-1500m) zones while the highlands (>1500m above sea level) had the lowest mold and aflatoxin

contamination (Kaaya and Kyamuhangire, 2006). Based on this categorization, the districts studied fall into the following groups ─ Mbarara (Western), highland; Iganga (Eastern), mid-altitude (moist); Gulu, Pader, and Kitgum (Northern), mid-altitude (dry) zone (Rwabwogo, 2007; Wortmann and Eledu, 1999). Hence, the environment and geography play key roles in mold growth, toxin production and dietary exposure in Uganda. Dietary intake of aflatoxins could also be affected by the types of food staples consumed in the region. For instance, consumption of plantain (locally referred to as *matooke*) is associated with lower dietary intake of aflatoxins (Asiki *et al.*, 2014). A recent report indicated that the Western region of Uganda has the highest production and consumption of *matooke* (Kilimo Trust, 2015) which could further explain the lower AFB_1 exposure levels in this population. There was no heterogeneity in the distribution of AFB_1-albumin adducts by sex, type of residence, and age group of the children. Thus, our results suggest that these factors do not affect the level of aflatoxin exposure in Ugandan children between 1 and 5 years of age. Our findings partly echoed those by Asiki and colleagues which did not observe any statistically significant differences by either age or gender of children but reported higher levels of AFB_1 adducts for communities closest to trading centers (Asiki *et al.*, 2014).

There are other factors that were not explored in this study that could potentially influence the extent of aflatoxin exposure in children. These may include the weaning status of children, seasonal effects, and dietary diversity (Wu *et al.*, 2014c). Previous studies have shown that exposure to potentially contaminated maize-based weaning foods could increase aflatoxin exposure (Gong *et al.*, 2003; Gong *et al.*, 2004). A recent study on child-feeding practices of primary caregivers in rural Uganda, highlighted early weaning as one of the major issues and that in some instances, children were weaned as early as 2 weeks and complementary foods including

maize-, millet-, cassava-, and *matooke*-based porridges were introduced (Nankumbi and Muliira, 2015). Previous studies have also demonstrated seasonal variations in the levels of aflatoxin exposure (Gong *et al.*, 2004; Mitchell *et al.*, 2016; Watson *et al.*, 2015). Dietary exposure to aflatoxins is usually higher during the dry season due to drought stress and elevated soil temperature which decrease the natural resistance mechanisms of plants to invasion by *A. flavus (Cotty and Jaime-Garcia, 2007; Diao et al., 2015)*. However, we do not expect our data to be affected by such variations since all blood samples were collected within the same season; the samples were collected between December 2015 and January 2016 which is a dry season in Uganda (Funk *et al.*, 2012). Finally, the quality of health care infrastructure and market access could buffer children against the nutritional challenges including dietary exposure to aflatoxins, that may be caused by seasonality and environmental changes (Shively, 2017).

In conclusion, the evidence discussed indicates the level of aflatoxin exposure among children in Uganda was very high and could potentially increase the risks of decreased child growth rate, immune dysfunction, and hepatocellular carcinoma later in life. Additional studies are needed to investigate and quantitate the relationship between aflatoxin exposure and child health.

CHAPTER THREE: Reduced foodborne toxin exposure is a benefit of improving dietary diversity

Data in this chapter has been published in Wu, F., Mitchell, N.J., Male, D. and Kensler, T.W., 2014. Reduced Foodborne Toxin Exposure is a Benefit of Improving Dietary Diversity. Toxicological Sciences 141(2): 329–334 (doi: 10.1093/toxsci/kfu137).

Abstract

Naturally occurring foodborne toxins are common in subsistence diets of low-income human populations worldwide. Often, these populations rely on one or two staple foods for the bulk of their calories, making them more susceptible to chronic intake of certain toxins. Exposure to common foodborne toxins is associated with diverse conditions such as cancer, immunotoxicity, growth impairment, and neurological deficits. Interventions focused solely on reducing toxin levels have proven difficult to sustain. Using case studies of two foodborne toxins, aflatoxin and cassava cyanide, this article addresses the heightened risk of particular diseases from eating monotonous diets based on maize, groundnuts, and cassava: common in sub-Saharan Africa and parts of Asia. We also discuss the potential role of increased dietary diversity in counteracting these diseases. Increased dietary diversity can reduce consumption of toxins and increase intake of nutrients that could counteract the toxicity of such chemicals. In Qidong, China, a population that previously consumed a monotonous maize-based diet and increased dietary diversity since the 1980s has experienced a dramatic reduction in liver cancer mortalities. That liver cancer decreased as dietary diversity increased is the catalyst for the hypothesis that dietary diversity could have a direct impact on reducing health effects of foodborne toxins. Future research, agricultural development, and food policy reforms should take into consideration the multifaceted benefits associated with improved dietary diversity. Collaborations between

toxicologists, nutritionists, and policymakers are important for development of sustainable interventions to reduce foodborne toxin exposure and promote health through increased dietary diversity.

Keywords: foodborne toxins, nutrition, diet, aflatoxin, cassava cyanide, global health

1. Introduction

Consumption of monotonous diets rich in carbohydrates but low in protein and micronutrients are common among poverty stricken communities. A profound public health benefit could be achieved through increased dietary diversity. Dietary diversity, a measure of the variety of individual food items or food groups consumed, has long been studied in the nutritional community in the context of health outcomes (Ruel and Menon, 2002). Generally, dietary diversity reflects dietary quality (Arimond and Ruel, 2004), adequacy of micronutrient intake (Moursi *et al.*, 2008), and dietary energy availability (Hoddinott and Yohannes, 2002). Low dietary diversity scores are related to poor nutritional status (Ey Chua *et al.*, 2012), a risk factor for multiple diseases common in low-income populations. Of particular importance is the dietary diversity and nutritional status of children residing in low-income countries where childhood death from malnourishment and disease are the highest. In 2004, 39% of child deaths were caused by micronutrient deficiencies, underweight, suboptimal breastfeeding, and preventable environmental risks (WHO, 2009).

In addition to improved micronutrient status through greater dietary diversity, another benefit that has received little to no attention is decreased human exposure to specific foodborne toxins. This concept is particularly important among populations in low-income nations who rely heavily on only a few dietary staples such as maize, groundnuts, cassava, and rice; which can be

highly contaminated with naturally occurring toxins. Such staples are often utilized in weaning children and can account for large portions of the diet throughout a person's lifetime. Therefore, exposure to foodborne toxins and the resulting health effects are often chronic in nature. Because cereals and grains make up the largest portions of the diet worldwide, toxins that naturally occur in these staples present the most risk for chronic disease. Fungal growth and infection of cereal and grain plants can result in contamination of food stuffs with various mycotoxins that are known carcinogens, immunosuppressants, teratogens, nephrotoxins and hepatotoxins (Richard *et al.*, 2003). Environmental conditions have a profound effect on fungal growth and subsequent mycotoxin contamination, making populations in certain climates more susceptible to exposure. The climatic conditions within tropical areas of the globe result in a high risk for mycotoxin contamination in both the field and during storage and handling, enhancing the difficulty for farmers and distributors to provide good quality grain products. The majority of areas where exposure to mycotoxins is continuous do not have the infrastructure and economic backing to sustain appropriate regulations, handling, and storage technologies to reduce mycotoxin contamination and consumption. In addition to mycotoxins, the environment in which certain staples are grown can affect levels of toxins such as metals. For example, consumption of large quantities of rice products by children has been considered to be the greatest exposure route for arsenic in children under 3 years of age (EFSA, 2009). Arsenic in water used on rice patties can accumulate in the grains causing various chronic effects over a lifetime, including cancer, skin lesions, neurologic, respiratory, cardiovascular, and developmental effects (Argos *et al.*, 2012; Barchowsky *et al.*, 2011; Naujokas *et al.*, 2013; Smith and Steinmaus, 2009). Plants also can naturally produce chemicals to protect themselves from consumption by pests, including humans. Some of these plants comprise tubers and roots, but can actually be eaten once properly

prepared and cooked. In areas where food insecurity and/or communities focus on a single cash crop for income, consumption of large quantities of potentially toxic or contaminated foods is seemingly unavoidable. Increasing both agricultural and dietary diversity could dilute the potential for chronic toxin consumption by such populations.

Increasing dietary diversity in these populations not only reduces exposure to foodborne toxins, but may allow for intake of dietary constituents that counteract the adverse effects of these toxins. Similarly, consumption of certain nutritional supplements such as folate, protein, selenium, etc., has been associated with decreased effects of other known food contaminants such as arsenic, lead, and cadmium. However, exposure to these toxins is known to occur through multiple routes and foods (Basu *et al.*, 2011; Gamble *et al.*, 2006; Gamble *et al.*, 2005; Hall *et al.*, 2009; Pilsner *et al.*, 2009; Steinmaus *et al.*, 2005) hence making the effect of dietary diversity on public health outcomes difficult to validate, and beyond the scope of this manuscript. Here, we describe how improved dietary diversity could directly and indirectly counteract the adverse health effects of two foodborne toxins: aflatoxin and cyanogenic glycosides. Additionally, we suggest potential public health benefits achieved through policy changes in agricultural biodiversity, global food security, and nutrition education in the developing world.

2. Aflatoxins

Aflatoxins are a group of chemically related toxins produced primarily by the foodborne fungi *Aspergillus flavus* and *A. parasiticus*. These fungi are particularly prevalent in tropical and subtropical regions, infecting food crops both in the field and postharvest. Aflatoxins, in particular AFB_1, contaminate multiple staple foods including maize and groundnuts. Roughly 5

billion people worldwide are exposed to uncontrolled aflatoxin contamination (Strosnider *et al.*, 2006). For decades, it has been known that AFB_1 exposure causes liver cancer in humans and a variety of animal species. The International Agency for Research on Cancer has classified naturally occurring mixes of aflatoxins as a Group 1 human carcinogen (IARC, 2002b). Currently, the United States Food and Drug Administration and the European Commission have set action limits of 20 and 4 μg/kg for food stuffs, respectively (FAO., 2004). Observed contamination of aflatoxin in maize from Africa and Southeast Asia often exceed these action levels. Based on estimated consumption of maize and peanuts, populations with the highest exposure risk could consume upward of 200 ng/kg body weight of aflatoxins per day making the risk for aflatoxin-induced hepatocellular carcinoma (HCC) in Africa and Southeast Asia range from 1740 to 17,300 cases annually (Liu and Wu, 2010). Additionally, concomitant exposure to aflatoxin and the hepatitis B virus (HBV) is common in developing countries, and has been shown to greatly increase liver cancer risk in Asia and Africa to upward of 78,000 cases per year (Kirk *et al.*, 2005; Liu *et al.*, 2012; Liu and Wu, 2010; Lunn *et al.*, 1997; Qian *et al.*, 1994; Ross *et al.*, 1992). There is also an increasing body of evidence that aflatoxins modulate the immune system (Jiang *et al.*, 2005; Jiang *et al.*, 2008; Turner *et al.*, 2003) and may lead to stunted growth in children (Gong *et al.*, 2002; Gong *et al.*, 2004; Khlangwiset *et al.*, 2011), potentially exacerbating health risks due to inadequate protein and micronutrient intake that predominates in impoverished communities.

Current strategies for reducing aflatoxin exposure in food include preharvest (strategies that reduce fungal infection of crops in the field), postharvest (improved drying and storage practices to prevent aflatoxin accumulation in stored food), and dietary interventions to enhance aflatoxin detoxification and elimination once ingested (Khlangwiset and Wu, 2010). However, many of

these strategies have not been sustainable over long periods of time and may not be economically feasible in poverty stricken communities. Less attention has been given to developing dietary diversity, which could reduce total aflatoxin exposure and its harmful effects.

A recent study has shown that decreased maize consumption, in favor of rice and other foodstuffs, has resulted in markedly lower aflatoxin exposures, and subsequently, dramatically declining liver cancer mortality rates in Qidong, China (Chen *et al.*, 2013). Prior to the 1980s, agricultural policies had restricted Chinese counties from trading food with one another, as food self-sufficiency was required of each county. Because the soil in Qidong was unsuitable for growing rice, maize was the primary staple grown, under environmental conditions that led to high aflatoxin contamination. As a result, Qidong became a "liver cancer hot spot," where 1 in 10 adult men died of liver cancer, often by age 45. When market reforms were introduced in the 1980s, the Qidongese increased their dietary diversity, turning from maize to rice and other foodstuffs from neighboring counties. As a result, aflatoxin exposure has plummeted in the last 30 years; correspondingly, the age-standardized rate of liver cancer mortality has decreased by 45% in the Qidong population since the 1980s (Chen *et al.*, 2013). This effect is independent of the recent deployment of a universal HBV vaccination program in China. Introducing dietary diversity into populations where aflatoxin-contaminated maize and groundnuts are dietary staples could not only reduce liver cancer risk, but could also reduce other adverse effects associated with aflatoxin exposure, such as growth faltering.

Hepatocarcinogenesis of AFB_1 is now a well understood mechanism that requires activation of the parent compound through oxidation by cytochrome P450s (Eaton and Gallagher, 1994; Ishii *et al.*, 1986). The intermediate product, aflatoxin-8,9-epoxide, is highly reactive and can form a

pro-mutagenic aflatoxin-N^7-guanine adduct within the DNA. Knowledge of the metabolic pathway and the mechanism of action provide a basis for modulation of aflatoxin's carcinogenic potential through chemo preventive agents available in foods. Improved dietary diversity could have the impact of introducing such protective phytochemicals (Kensler *et al.*, 2004). Compounds found in cruciferous vegetables, onions, and garlic can reduce aflatoxin-induced cancer by enhancing glutathione-S-transferase (GST) expression, which mediates conjugation of the reactive intermediate aflatoxin-8,9-epoxide and diverts its interaction with DNA. Randomized clinical trials utilizing a broccoli sprout beverage rich in the phytochemical sulforaphane demonstrated protective alterations in aflatoxin excretion (Kensler *et al.*, 2011). Chlorophyllin, a derivative of chlorophyll, is a natural constituent of green vegetables in the human diet that has shown anti-carcinogenic effects in animals (Dashwood *et al.*, 1998). Chlorophyllin appears to protect against aflatoxin by sequestering aflatoxin during the digestive process and hence impeding aflatoxin's absorption (Egner *et al.*, 2001).

Because aflatoxin is one of the most important risk factors for one of the deadliest cancers worldwide, liver cancer, reducing its presence in human diets is critical. Possibly even more critical from a global health standpoint is the potential association between aflatoxin exposure and growth impairment in children, which can lead to a variety of adverse health conditions that last well beyond childhood.

3. Cyanogenic glycosides

The presence of cyanogenic glycosides in bitter cassava (cassava cyanide) presents another example in which increasing dietary diversity may not only reduce consumption of the risky foodstuff, but may provide nutrients that biochemically counteract the foodborne toxins. The

Food and Agriculture Organization (FAO) estimates that cassava comprises an essential part of the diet for 500 million people worldwide (Phillips *et al.*, 1999).

Cassava, particularly the bitter variety grown in resource-poor settings, contains cyanogenic glycosides that protect the root against pest consumption. Based on developmental toxicity studies with the major cyanogenic glycoside found in cassava, linamarin, the Joint FAO/WHO Expert Committee on Food Additives and Contaminants (JECFA) set an acute reference dose (ARfD) of 90 μg/kg body weight for cyanide equivalents. Chronic consumption of cassava, which can contain linamarin from 15 to 1000 mg/kg, can result in adults exceeding the ARfD by a threefold factor (Feeley *et al.*, 2012). To reduce cyanide, cassava root is typically processed by sun drying or soaking. These traditional practices could take up to 7 days to reduce glycosides to levels safe for human consumption. Unfortunately, during times of food shortages, cassava processing may be shortened or eliminated (Boivin *et al.*, 2013).

Cassava cyanide exposure can lead to a variety of adverse neuromuscular and neurocognitive effects. Konzo in particular is an ongoing public health concern in poor areas dependent on cassava. It often occurs in the most remote areas of Africa, leading to a lack of attention by health authorities and the research community. A paralytic disease, konzo is characterized by a permanent, non-progressive loss of motor function typically in the legs. Severe cases can result in a lifetime inability to walk. The loss of motor function is caused by exposure to cyanogenic glycosides in cassava causing selective upper motor neuron damage.

A recent publication shows that neurocognitive deficits can occur even in children who do not develop konzo, if they are living in a konzo-endemic region (Boivin *et al.*, 2013). In the Democratic Republic of Congo, children with konzo performed the most poorly on

neurocognitive and neuromotor tests; but even children without konzo who lived in a konzo-endemic region performed significantly more poorly than children not living in an endemic area. This implies that in high-risk regions, average exposures to cassava cyanide that may not result in konzo can still cause other adverse cognitive effects in children.

The neurotoxic mechanism for cyanogenic glycosides has not yet been clarified, however, there is evidence that the effects occur either within the corticomotor neurons or the descending motor pathways (Tshala-Katumbay *et al.*, 2002; Tylleskär *et al.*, 1993). Cyanogenesis occurs in cassava when the plant tissue is damaged and initiation of this process involves the conversion on linamarin by linamarase to acetone cyanohydrin. Acetone cyanohydrin in cassava is unstable and will decompose to acetone and hydrogen cyanide at pH >5 and/or at temperatures exceeding 35°C (Nzwalo and Cliff, 2011). The high pH within the gut is hypothesized to induce the conversion of acetone cyanohydrin to cyanide, thus causing cyanide toxicity and ultimately konzo and other neurological diseases.

Yet not everyone who consumes cassava worldwide is at risk of developing konzo or neurocognitive deficits. Konzo is associated with the combination of specific conditions: dependence on cassava in the diet, low sulfur amino acid intake, and conditions of hardship (drought, famine, conflict) that drive populations to consume inadequately processed cassava (Nzwalo and Cliff, 2011). In particular, the combination of low sulfur amino acid intake with high cassava intake appears to play a role in all major konzo epidemics recorded (Banea *et al.*, 2013). Detoxification of cyanide within the body involves transformation of cyanide into thiocyanate by the enzyme thiosulfate cyanide transsulfurase which requires sulfur donors. Dietary sulfur amino acids, which are deficient in cassava, provide protective capacity from

cyanide toxicity through promoting detoxification pathways and excretion of thiocyanate in the urine (Nzwalo and Cliff, 2011; Tor-Agbidye *et al.*, 1999). Improving dietary diversity, where possible, can reduce two of these three risk factors. It will not only reduce the total amount of bitter cassava consumed per individual hence, reducing the burden of cyanogenic glycoside intake but could also introduce sulfur, which aids in detoxification of cyanide in the body. Dietary sulfur amino acids from protein sources eggs, dairy, meat, and legumes are important in the detoxification of cyanide in the body.

4. Conclusions

Dietary diversity and foodborne toxins have traditionally been studied in separate fields: nutrition and toxicology. It is important to consider their interactions, which are relevant to the health of billions of the most vulnerable human populations worldwide. Nutritionists with a focus on global health should consider that another benefit of increasing dietary diversity is to reduce the overall burden of foodborne toxins and to counteract these toxins' adverse effects in the body. Food toxicologists who develop interventions to reduce toxin levels in food crops should consider the enhancement of dietary diversity among their suite of interventions. Dietary diversity promotion and education in poor populations and among research scientists should focus on this dual benefit in order to optimize interventions and research programs.

We have described two foodborne toxins common in monotonic diets: aflatoxin and cyanogenic glycosides. Not only would increasing dietary diversity in populations where maize, groundnuts, and cassava decrease the overall intake of these toxins; it would also increase the likelihood of consuming nutrients that could counteract their adverse effects to improve health. Thus, governmental programs that make production of diverse crops profitable to farmers and diverse

foodstuffs accessible to consumers should be considered a priority in developing countries. Such governmental policy changes were shown to be effective in reducing both aflatoxin exposure and consequently primary liver cancer in the Qidongese in China (Chen *et al.*, 2013).

Education in rural areas and developing nations on the health impacts of a diverse diet through advertising, marketing, and public health workshops are needed to inform communities of the long-term benefits of consuming a diverse diet and, likewise, potential risks to human health of a monotonous diet. Additionally, governmental policies and education should focus on added benefits of introducing agricultural biodiversity, like decreased residual pesticide exposure, as a means of inciting interest in the population and incentivizing societies to maintain such practices. Educating farmers on the benefits to soil health and crop yield, thus increasing their profits, through rotation and intercropping systems would not only benefit the farmers but would influence dietary diversity and population health as well. Development of more diverse cropping systems has proven to yield similar profit margins as mono-cropping systems while also decreasing external synthetic inputs such as pesticides, herbicides, and fertilizers. Thus, diverse agriculture systems can lower residual pesticide contamination of freshwater and soil that can negatively impact human health (Davis *et al.*, 2012; Lechenet *et al.*, 2014).

Improving human health through dietary diversity will involve a multilevel approach that influences agricultural production, extension, marketing, education, and policy development (Frison *et al.*, 2006). The International Plant Genetic Resources Institute has been working to increase dietary diversity in developing areas for several years, and has identified multiple challenges to the diversification and use of "neglected and underused species" of crops. Included in these challenges is a lack of investment by funding agencies in research projects with

comprehensive nutrition goals (Frison *et al.*, 2006). The vast majority of dietary intervention strategies focus on increases of a single dietary nutrient, such as protein, vitamin A, or iron. Singularly focused interventions may have limited efficacy in sustainably improving population health.

Changes in food consumption practices are difficult to achieve in populations, as these practices are often passed down from generation to generation, and food is a central part of many cultures. Improving dietary diversity, however, does not necessitate a complete change in food traditions; rather, augmentation of dietary staples with additional foodstuffs as regularly as possible. Initiatives to increase crop biodiversity in developing countries have the benefit of improving nutrition and decreasing exposure to foodborne toxins and carcinogens. This dual impact needs to be emphasized in policy decision-making among global agricultural and public health communities.

CHAPTER FOUR: Modeling the anorectic potencies of food-borne trichothecenes by benchmark dose and incremental area under the curve methodology

Data in this chapter has been published in Male, D., Mitchell, N., Wu, W., Bursian, S., Pestka, J. and Wu, F., 2015. Modelling the anorectic potencies of food-borne trichothecenes by benchmark dose and incremental area under the curve methodology. World Mycotoxin Journal 9 (2): 279-288. (doi: http://dx.doi.org/10.3920/WMJ2015.1961).

Abstract

Fusarium spp. fungi produce a spectrum of trichothecene mycotoxins that often simultaneously contaminate cereal grains. These have the potential to contribute jointly to adverse effects such as anorexia and emesis. For the purposes of risk assessment and regulation, it is desirable to assign toxic equivalency factors (TEFs) to each of these trichothecenes, as has been successfully done for anthropogenic toxicants such as polyhalogenated aromatic hydrocarbons. As a first step towards this end, we employed a mouse model to compare the anorectic potencies of deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), nivalenol, fusarenon-X (FX), T-2 and HT-2 toxin (T-2 and HT-2) following oral exposure by gavage using two approaches. In the first approach, the US Environmental Protection Agency (US-EPA) benchmark dose (BMD) method for continuous data was used to calculate the BMD relative to DON 2 h after dosing. The order of potency based on BMD values was: DON (1) ≈ 3-ADON (1) ≈ 15-ADON (1) < NIV (3) < HT-2 (5) < FX (9) << T-2 (124). In a second approach, time course effects of each toxin at fixed doses were measured by calculating the incremental area under the curve (IAUC) over 16 h. DON caused significant feed refusal within the first 30 min after exposure, lasting only 3 h while for 3-ADON and 15-ADON, feed refusal lasted 6 h. NIV, FX, T-2, and HT-2 toxins caused the longest duration of feed refusal,

lasting up to 16 h. Based on IAUC values, the order of relative potency was as follows: DON (1) < 3-ADON (2) ≈ 15-ADON (2) < NIV (7) < FX (10) << T-2 (31) < HT-2 (34). These results provide a foundation for developing consensus TEFs that will be amenable to future risk assessment of trichothecene mixtures.

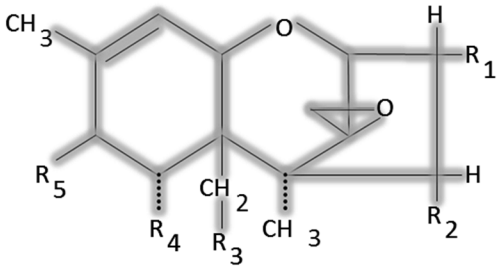
Keywords: deoxynivalenol, anorexia, toxic equivalency factor

1. Introduction

1.1 Foodborne trichothecenes occur as mixtures

Fusarium spp. has the potential to produce an array of trichothecene mycotoxins in cereal grains and resultant foods. Exposure to trichothecenes causes multiple adverse effects in experimental animals including anorexia, emesis and growth suppression (Escriva *et al.*, 2015; Pestka, 2010a; Wu *et al.*, 2010). Common molecular mechanisms for this mycotoxin family include interference with cellular translation by binding to eukaryotic ribosomes as well as activation of stress pathways via intracellular protein kinases, G protein-coupled receptors and transient receptor potential (TRP) ion channels (Pestka, 2010a); (Zhou and Pestka, 2015). The trichothecenes found in food are classified as Type A and Type B based on their chemical structures as presented in Figure 4. The Type A group includes T-2 and HT-2 toxins, while the Type B group includes deoxynivalenol (DON), nivalenol (Supriya *et al.*), and their acetylated derivatives (3-ADON, 15-ADON, and fusarenon-X (FX)) (Gale *et al.*, 2011a). The double bond between C-9 and C-10 and the epoxy ring between C-12 and C-13 are primarily responsible for the toxicity of trichothecenes (Wu *et al.*, 2013c). Several studies have shown that removal of these groups resulted in complete loss of toxicity in mammalian cells (Abbas *et al.*, 2013; Wu *et al.*, 2013c).

Substitutions at the R1, R2, R3, R4, or R5 in trichothecene congeners can greatly modulate their toxicity.



Trichothecene	R1	R2	R3	R4	R5
Deoxynivalenol	OH	H	OH	OH	=O
3-acetyldeoxynivalenol	OAc	H	OH	OH	=O
15-acetyldeoxynivalenol	OH	H	OAc	OH	=O
Nivalenol	OH	OH	OH	OH	=O
Fusarenon-X	OH	OAc	OH	OH	=O
T-2 toxin	OH	$OCOCH_3$	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$
HT-2 toxin	OH	OH	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$

Figure 4. Chemical structure of simple trichothecenes

DON, the most commonly occurring trichothecene in cereals worldwide, is also known as "vomitoxin" due to its induction of vomiting in pigs. DON is produced mainly by *Fusarium graminearum* and *F. culmorum* (Kosawang *et al.*, 2014). The key feature that differentiates DON and its derivatives from other trichothecenes is the lack of a hydroxyl functional group at the C-4 position. Acetylation of DON at the C-3 and C-15 positions results in 3-ADON and 15-ADON, respectively. Co-occurrence of these trichothecenes has been reported in numerous cereal grains including contaminated maize grains for human consumption that contained 11.7-13.7% of DON, 15% of 15-ADON, and 0.9-7.2% of 3-ADON (EFSA, 2013a). The acute reference dose (ARfD) of 8 µg/kg bw/day and a tolerable daily intake (TDI) of 1 µg/kg bw/day were

recommended by the Joint FAO/WHO Expert Committee on Food Additives as limits for human exposure to mixtures of DON, 3-ADON, and 15-ADON in food (JECFA, 2010).

NIV is produced mainly by *Fusarium cerealis* and *F. poae,* and to a lesser extent by *F. graminearum* and *F. culmorum* (Malachova *et al.*, 2014b). The chemical structure of NIV is similar to that of DON; however, it contains a hydroxyl group at the C-4 position of the trichothecene rings. Co-occurrence of NIV with DON in maize, wheat, barley, and oats has been reported (Gale *et al.*, 2011b; Malachova *et al.*, 2014a). FX naturally co-occurs with NIV in cereals (Saengtienchai *et al.*, 2014). FX is biochemically similar to NIV with an acetyl group at C-4 as opposed to a hydroxyl group. It may be produced directly by fungi or result from acetylation of NIV in the liver (Saengtienchai *et al.*, 2014). Currently, the European Food Safety Authority recommends a joint TDI for NIV equal to 1.2 µg/kg bw/day (EFSA, 2013b; Malachova *et al.*, 2014b).

T-2 and HT-2 toxins are mainly produced by *Fusarium sporotrichoides and F. langsethiae*. These toxins may also be produced to a lesser extent by *F. equiseti, F. poae,* and *F. acuminatum* (Yoshinari *et al.*, 2014). T-2 and HT-2 toxins co-occur in most grain and grain products of wheat, maize, barley, rice, soybeans, and oats grown in cooler regions of the world. T-2 toxin is known as one of the most toxic mycotoxins, capable of causing sub-lethal and lethal toxicosis. T-2 has also been associated with alimentary toxic aleukia (ATA) in humans (Joffe, 1974) and is classified as a biological weapon (Kuca and Pohanka, 2010). Acute exposure to T-2 and HT-2 toxins cause food and feed refusal, emesis, neurotoxicity, leucocytosis, skin irritation, haemorrhage, and immune modulation (Weidner *et al.*, 2013). T-2 can be metabolized to HT-2 within the liver (Krska *et al.*, 2014). Because of the similar toxic effects caused by these toxins, JECFA established a joint regulatory limit; the provisional maximum tolerable daily intake

(PMTDI) 60 ng/kg bw/day in humans for either T-2 or both T-2 and HT-2 toxin (Wu *et al.*, 2014a).

1.2 Can a toxic equivalency factor approach be applied to simple trichothecenes?

While DON is the predominant contaminant trichothecene found in common cereals such as wheat and barley (Bertuzzi *et al.*, 2014a; Yoshizawa, 2013), it is difficult to make generalizations about its occurrence relative to other trichothecenes in food since the *Fusarium* profile is significantly affected by multiple factors, including climate, seasonal changes, and geographical region of production (Ibanez-Vea *et al.*, 2012). Toxic equivalency factor (Bianco *et al.*) methodology refers to the expression of toxicity of one or more compounds in relationship to a reference compound, as a means to properly assess risk of complex mixtures of compounds that typically co-occur, share common mechanisms of action, and have additive effects (Van den Berg *et al.*, 1998a; Van den Berg *et al.*, 2006a). The TEF concept was developed to facilitate risk assessment and regulatory control of polychlorinated dibenzodioxins (PCDDs), polychlorinated biphenyls (PCBs), and polychlorinated dibenzofurans (PCDFs) (Van den Berg *et al.*, 1998a; Van den Berg *et al.*, 2006a). In the environment, these groups of anthropogenic contaminants often occur as mixtures; thus, humans are exposed to multiple congeners simultaneously. These compounds have structural similarities, share the same mode of action (Simsek *et al.*, 2013), and can persist and accumulate in the food chain. In the case of the PCDDs, PCBs, and PCDFs, the individual toxicity of each compound has been calculated and represented as a consensus order of magnitude TEF in comparison to the selected reference compound, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Van den Berg *et al.*, 1998a). To determine the toxicity of a mixture of TCDD-like chemicals, the concentration of each compound is multiplied by its TEF, the quotient of which is expressed as toxic equivalents (TEQs), which is the compound's

individual contribution towards the overall toxicity of the mixture. The TEF method assumes that the effects of TCDD and TCDD-like compounds are additive, thus the TEQs contributed by each compound are summed to give the total TEQ (or TCDD-like) concentration of the mixture.

The interactions between trichothecenes in mixtures are yet to be fully understood. There are a handful of studies that have considered this issue, but these mostly focus on DON, NIV, and T-2 toxin (Bianco *et al.*, 2012; Tajima *et al.*, 2002; Thuvander *et al.*, 1999) but not their derivatives. In an in vitro study to determine inhibition of DNA synthesis, Tagima and colleagues (2002) found that trichothecene mixtures containing DON, T-2, and NIV exhibited both synergistic and additive effects at low doses but not at the highest doses (Tajima *et al.*, 2002). Similar results were reported for trichothecene mixtures containing NIV and either DON, Diacetoxyscirpenol DAS, or T-2 toxin (Thuvander *et al.*, 1999). However, this study also reported slight antagonism for pairs of toxin mixtures containing DON and either DAS or T-2 toxin (Thuvander *et al.*, 1999) while another study found neither additive nor synergistic effects (Bianco *et al.*, 2012). Although the properties of trichothecene interactions are still questionable they contain other properties that would make determination of values similar to TEFs feasible and valuable to the risk assessment industry. Trichothecenes typically co-occur within the environment (Šegvić Klarić, 2012), are structurally analogous, and share a common mode of action (Pestka *et al.*, 2004) making them good candidates for the application of a relative potency methodology similar to that of the TEFs developed for TCDD-like chemicals.

Here, we have completed the first steps to develop the TEF concept for the trichothecene mycotoxins to establish a more comprehensive understanding of the toxicological risk from exposure to complex mixtures of these naturally occurring compounds (Pestka *et al.*, 2004). Towards this goal, we analysed existing published toxicological data for Type A and Type B

trichothecenes derived from a well-characterized murine food refusal bioassay (Wu *et al.*, 2014d; Wu *et al.*, 2015) through two methodologies: (1) US-EPA benchmark dose (BMD) method for continuous data and (2) calculation of total anorectic effect based on incremental area under the curve (IAUC) (Wolever *et al.*, 1991). Other metrics could have been used in place of the BMD including the lethal dose to 50% of the study population (LD_{50}), the no observed effect level (Strosnider *et al.*) or the lowest observed effect level (LOEL). However, we chose the BMD because this is the most current metric used as the point of departure in risk assessments documented by numerous regulatory risk assessment bodies worldwide; including the Joint Expert Committee on Food Additives (JECFA) of the Food and Agriculture Organization and World Health Organization, the European Food Safety Authority (EFSA), and the United States Environmental Protection Agency (US-EPA). Further, we calculated and used the IAUCs to acknowledge the importance of duration of toxic effect, in ranking the relative toxicities of the trichothecenes; which cannot be captured purely by a measurement in a single point in time. Utilizing the BMD and IAUC values, we calculated the relative potencies of each trichothecene in relation to DON. These potency data will be applicable to the future development of consensus TEFs and calculation of TEQs for trichothecenes, ultimately facilitating better risk assessment and a more comprehensive approach to international regulatory action limits for these toxins in foods.

2. Materials and methods

The raw toxicological data of trichothecene-induced feed refusal in mice used for this analysis were obtained from previously published research (Wu *et al.*, 2012b; Wu *et al.*, 2014d; Wu *et al.*, 2015). Using feed refusal as the endpoint, two methods were used to assess toxic potential of DON, 3-ADON, 15-ADON, NIV, FX, T-2 and HT-2 following oral exposure. First, BMD

modelling at a static endpoint was used to ascertain points of departure. Second, to assess toxicity over time, we calculated total effect based on IAUC from onset to remediation of food refusal. Relative potencies for both BMD and IAUC modelling were calculated relative to DON. We employed DON as the reference compound; similar to how TCDD is used as the reference compound for dioxin TEF methodology for two reasons: (1) the relative abundance of DON among the trichothecenes in cereal grains worldwide; and (2) the amount of scientific knowledge available on its toxicity and mechanism of action.

2.1 Benchmark dose modeling

The BMD represents the dose in a toxicological dose-response curve that corresponds to a particular proportion of the study population experiencing an adverse response; this is known as the benchmark response value (BMR) and is typically set at 10% of the population (BMD_{10}). For risk assessment purposes, the 95% lower confidence limit of this BMD, called the BMDL, is used as a 'point of departure,' to divide by uncertainty factors to arrive at a safe daily dose for humans (a 'reference dose' or 'tolerable daily intake'). The BMD modelling approach involves fitting pre-existing mathematical models to actual dose response data or results generated from toxin exposure studies. For each trichothecene described above, a BMD and BMDL was calculated based on dose-response curves generated in mouse feed refusal studies, where the toxicity (feed refusal) was measured 2 h following exposure.

BMD analyses were conducted using the US-EPA official Benchmark Dose Software version 2.40 (BMDS240). The protocols for the anorectic animal studies can be found in recent publications by Wu and co-workers (Wu *et al.*, 2012b; Wu *et al.*, 2014d; Wu *et al.*, 2015). The mean, standard deviation, and standard error for feed consumption of each toxin dose observed 2 h following gavage administration constituted the raw data that were utilized in the BMDS240

software. The BMDs for the trichothecenes were estimated using the method for continuous data. For continuous data, where there is not a commonly known and/or accepted level of response to consider adverse, a change in the mean of the treatment group equal to one control standard deviation from the control mean is used to assign a BMR value, and consequently calculate the BMD and BMDL in relation to that BMR (Crump, 1995). Because there is no accepted level of response to consider significantly adverse in relation to feed refusal resulting from trichothecene exposure we did not set the BMR and BMD to 10% of the population, but instead utilized the value as described above; the value where the change in the mean of the treatment group was equal to one control standard deviation from the control mean. In our study, feed consumption has an inverse relationship with toxin dose and thus, produced a decreasing data trend in the BMD modelling. The models that best described the data were selected based on the Akaike Information Criterion, BMD to BMDL ratio, BMDL value, P-values, and visual inspection of graphical outputs; which provide information on the goodness of fit of each statistical model to the dose-response data points (US-EPA, 2012). The BMD values of all trichothecenes following 2 h of exposure were determined, and their respective relative anorectic potencies were calculated as a ratio of the BMD of DON to BMD of each toxin.

2.2 Incremental area under the curve

The IAUC has been previously used in studies to measure blood composition changes following nutrient intake among diabetic patients. For example, IAUC was strongly correlated with glycemic index changes in response to glucose intake (Brand-Miller *et al.*, 2009; Kjøllesdal *et al.*, 2014; Le Floch *et al.*, 1990). The IAUC was also correlated to triacylglyceride level when an oral fat load was given to individuals with type II diabetes (Carstensen *et al.*, 2003). The glycemic index is defined as 'the incremental area under the blood glucose response curve

(AUC) after consumption of a 50g available carbohydrate portion of a food expressed as a percentage of that after 50g of standard oral glucose' (Brand-Miller *et al.*, 2009; Kjøllesdal *et al.*, 2014).

Feed consumption data for multiple time points following oral administration of a fixed oral dose of 2.5 mg/kg bw of DON, 3-ADON, 15-ADON, NIV, and FX were used to assess the magnitude and duration of anorectic effects in the mouse model. These data were used to compare anorectic dose response effects of DON and its congeners on cumulative feed intake (Wu *et al.*, 2014d). A similar analysis was conducted for T-2 and HT-2 using feed consumption data for the 0.5 mg/kg bw dose over multiple time points. The basal mean feed intake at the respective time points was also determined using a control group. Both the 2.5 mg/kg bw and 0.5 mg/kg bw doses were higher than the estimated BMD values for the respective trichothecenes in this study. Mean feed consumption was calculated by time for each toxin and compared to control animals. The plot of feed intake against time for both controls and exposed animals yield consumption curves. The area under each curve was calculated using the trapezoid rule described in several other clinical studies (Brand-Miller *et al.*, 2009; Le Floch *et al.*, 1990).

$$AUC = ½ \times \Delta T \times (\text{Feed intake A} + \text{Feed intake B})$$

where A and B are initial and final feed consumption for each period, and ΔT is change in time or duration between A and B.

To estimate feed refusal in mice as a result of exposure to trichothecenes, the difference between the AUC for controls and toxin-treated groups was calculated. This difference is the incremental area under the curve. The differences are negative values that represent decreases in feed consumption since the IAUC of controls is higher than that of exposed animals. The total IAUC

for each toxin was calculated as the sum IAUCs of all segments of the curve from time point zero to the time point at which animals began to recover from the anorectic effects. Relative potency values based on IAUC were calculated as a ratio of the total IAUC of DON to each mycotoxin administered. It is important to note that the onset and duration of effects were different for each mycotoxin and consequently, the IAUC values are based on varying lengths of time. Additionally, the IAUC values were multiplied by a factor of 5 for T-2 and HT-2 for comparison with DON, due to the difference in doses.

3. Results

3.1 Relative potency of trichothecenes based on benchmark dose

In Table 3, the BMD values following oral and intraperitoneal exposure are summarized for all the trichothecene mycotoxins tested. The relative potency values were calculated as the ratio of the BMD of DON to the BMD value of each of the other toxins. The BMD values are inversely related to the potency of a substance. A higher BMD value means that a higher concentration of a toxic material is required to elicit a significant response or change in the anorectic effect, thus making its overall potency lower. As expected, T-2 is the most potent of all the tested trichothecenes. Based on the BMDs calculated for these 7 mycotoxins via oral exposure, the route most significant to human exposure, the order of toxicity is DON ≈ 3-ADON ≈ 15-ADON < NIV < FX < HT-2 << T-2.

Exposure of mice to 0.5, 1.0, 2.5, and 5.0 mg DON/kg bw resulted in 31, 21, 68, and 77% reduction in feed intake after 2 h, as compared to the controls. The BMD was calculated as 1.5 mg/kg bw/day. Acute exposure of mice to 0.25, 1.0, 2.5, and 5.0 mg of 3-ADON/kg bw by gavage reduced feed intake by 5, 18, 66, and 83%, respectively, after 2 h. The BMD of 3-ADON

determined at the time of peak effect was 1.3 mg/kg bw/day. When reduction in feed intake following oral exposure of mice to 0.5, 1.0, 2.5, and 5.0 mg/kg bw of 15-ADON was measured, 15-ADON decreased feed consumption by 11, 5, 38, and 60%, respectively. The BMD for 2 h gavage exposure was calculated as 1.2 mg/kg bw/day. When mice were exposed to 0.01, 0.1, 1.0, and 5.0 mg/kg bw of NIV per day by gavage, the amount of feed consumed after 2 h decreased by 7, 12, 28, and 44% compared to the control group, respectively. The BMD calculated was 0.5 mg/kg bw/day. The amount of feed consumed 2 h after administering 0.025, 0.25, 1.0, and 2.5 mg/ kg bw of FX toxin to mice by gavage was reduced by 13, 27, 40, and 53%, respectively. The BMD calculated was 0.2 mg/kg bw/day. Thus, FX was more potent than DON or NIV.

Table 3. Summary of benchmark dose (BMD) and relative potencies calculated at 2 h after administration (gavage) of trichothecenes

Trichothecene	BMD (mg/kg bw)	Relative potency
DON	1.5	1
3-ADON	1.3	1
15-ADON	1.2	1
NIV	0.5	3
FX	0.2	9
T-2	0.01	124
HT-2	0.3	5

DON = deoxynivalenol; ADON = acetyldeoxynivalenol; NIV = nivalenol; FX = fusarenon-X; T-2 = T-2 toxin; HT-2 = HT-2 toxin.

Feed refusal following oral dosing of 0.01, 0.1, 0.5, and 1.0 mg/kg bw of T-2 after 2 h as 24, 46, 47, and 57%, respectively, as compared to controls. The BMD calculated was 0.01 mg/kg bw/day, equivalent to a potency of 124 relative to DON. Oral administration of 0.01, 0.1, 0.5,

and 1.0 mg/kg bw of HT-2 resulted in 17, 22, 69, and 68% reduction in feed intake as compared to controls, respectively, after 2 h, The BMD value calculated was 0.3 mg/kg bw/day and thus the potency relative to DON was 5.

3.2 Relative potency of trichothecenes based on incremental area under the curve

Figure 5 shows the kinetics of food refusal response in relation to the control group for each of the Type B trichothecenes over a 16 h period following oral administration of 2.5 mg/kg bw/day. The IAUC increases initially as long as animals experience anorectic effects, until a time when they recover and consume more feed than the control animals (Table 4). At that point, the IAUC decreases and serves as an indicator used to measure duration of toxic effects. The percent decreases in feed intake during each of the time periods between 0.5 to 16 h after administration of each mycotoxin are shown in Table 5. The time at which the percent decrease becomes negative indicates a recovery from anorexia within a specific treatment group.

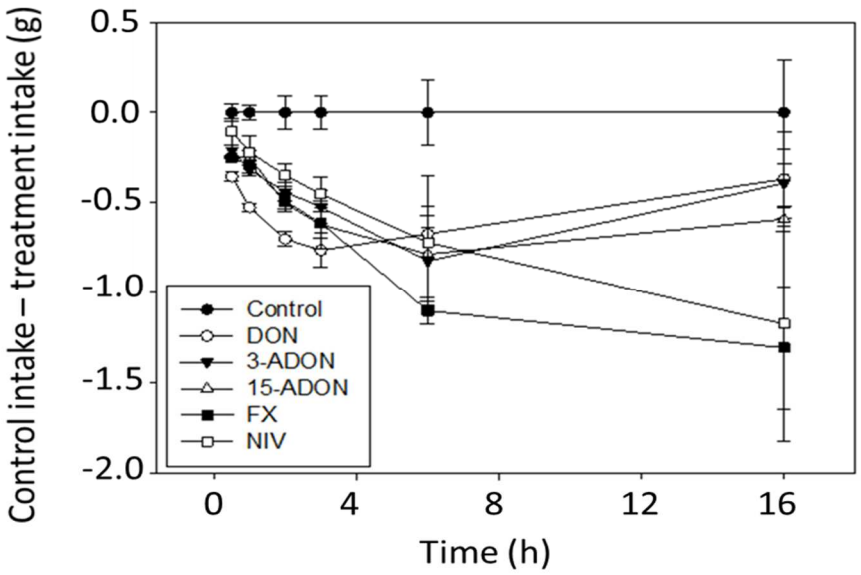


Figure 5. Time course relationship of reduced feed consumption compared to control group for five type-B trichothecenes. Mice were orally dosed with either phosphate buffered saline (control group) or 2.5 mg/kg bw of the specified toxin. NIV = nivalenol; FX = fusarenon-X; DON = deoxynivalenol; 3-/15-ADON = 3-/15-acetyldeoxynivalenol. Data are presented as mean ± standard error of the mean (n=5/group) (Wu *et al.*, 2014d).

DON, 3-ADON, and 15-ADON caused significant decreases in feed intake within 0.5 h, post exposure, as compared to controls. The anorectic effects lasted about 3 h for DON and 6 h for 3-ADON and 15-ADON, respectively (Figure 5). Oral dosing of NIV resulted in a delayed response; its peak reduction in food intake, compared with controls, occurred at 6 h following exposure (Table 5). However, the anorectic effects of NIV lasted over 16 h, with the animals never recovering from the toxicity within the experimental limits. Treatment of mice with FX toxin caused both rapid and long-term feed refusal. Within the first 0.5 h, FX caused 92% reduction in feed intake as compared to controls and, similar to NIV, the anorectic effects lasted over 16 h. Based on the IAUC calculations, oral administration of FX toxin caused the highest total decrease in feed consumption of all Type B trichothecenes studied.

Table 4. Comparison of IAUCs for food intake suppression after oral exposure to type B trichothecenes

		IAUC (g.h)				
Time Point (h)	Duration ΔT (h)	DON	3-ADON	15-ADON	NIV	FX
0.5	0.5	0.178	0.105	0.125	0.053	0.127
1	0.5	0.441	0.264	0.25	0.163	0.272
2	1	0.614	0.379	0.377	0.284	0.39
3	1	0.733	0.482	0.563	0.400	0.552
6	3	0.719	0.674	0.704	0.587	0.858
16	10	0.521	0.608	0.689	0.949	1.205

Mice were dosed by oral gavage with 2.5 mg/kg bw of each toxin. DON = deoxynivalenol; ADON = acetyldeoxynivalenol; NIV = nivalenol; FX = fusarenon-X. Increasing IAUC values indicate an increase in toxic effect. A decrease in IAUC value from one time point to the next indicates a 'recovery' from the anorexic effect.

Table 5. Comparison of percent feed refusal for type B trichothecenes as determined from incremental area under the curve

		Decrease in feed intake per period (%)				
Time Point (h)	Duration ΔT (h)	DON	3-ADON	15-ADON	NIV	FX
1	0.5	89.5	62.8	0	50	28.1
2	1	78.6	65.6	90.7	41.3	87.7
3	1	28.2	23.6	35.3	33.5	56.4
6	3	-10.8	35	21.1	27.5	69.7
16	10	-20.9	-25.4	-11.2	23.7	11.4

Percent feed refusal was calculated as the difference in the amount of food consumed by animals given 2.5 mg/kg bw of type B trichothecene by oral gavage and the control group between time points. A negative value indicates a recovery from the trichothecene toxicity and larger food consumption than controls.

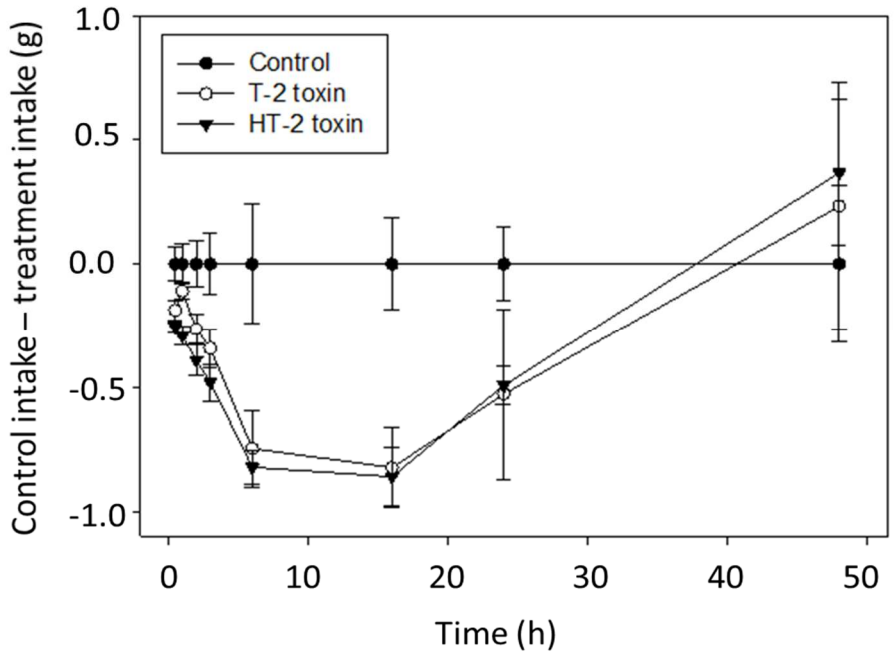


Figure 6. Time course relationship of reduced feed consumption compared to control group for T-2 and HT-2 toxins. Mice were orally dosed with either phosphate buffered saline (control group) or 0.5 mg/kg bw of the specified toxin. Data are presented as mean ± standard error of the mean (n=6/group) (Wu *et al.*, 2015).

Figure 6 shows the kinetics of food refusal response for each of the Type A trichothecenes over 48 h following oral administration of 0.5 mg/kg bw. The feed consumption results at various time points following gavage administration of T-2 and HT-2 were used to calculate both IAUC (Table 4) and percent feed refusal (Table 5) to determine the magnitude of anorectic effect. The feed intake of control animals is used to calculate percent decrease in feed intake during each of the time periods between 0.5 to 48 h. Both T-2 and HT-2 caused a greater than 60% reduction in feed intake within 0.5 h post exposure (Table 5). The effects of oral exposure of mice to T-2 and HT-2 lasted 16 h, similar to that of FUS-X and NIV trichothecenes. Time course analysis of the effects of both T-2 and HT-2 showed that decreases in feed intake peaked around 16 h, after which animals began to recover from the anorectic effects (Tables 6 and 7).

Table 6. Estimated incremental area under the curve (IAUC) for food intake suppression after oral exposure to type A trichothecenes

Time Point (h)	Duration ΔT (h)	IAUC (g.h)	
		T-2	HT-2
0.5	0.5	0.094	0.128
1	0.5	0.203	0.274
2	1	0.241	0.341
3	1	0.303	0.435
6	3	0.545	0.651
16	10	0.785	0.841
24	8	0.675	0.676
48	24	0.147	0.062

Mice were dosed by oral gavage with 2.5 mg/kg bw of each toxin. Increasing IAUC values indicate an increase in toxic effect. A decrease in the IAUC value from one time point to the next indicates a 'recovery' from the anorexic effect.

Animals began to consume higher total amounts of feed after about 24-40 h post-treatment than the control group when dosed orally. The information generated from both IAUC and percent feed refusal is used jointly to determine the magnitude of the anorexic effect. Potency values relative to DON were calculated for each trichothecene analyzed based on the total AUC for feed refusal over time. It is important to note that the AUC calculated for this comparison, which we have termed the 'effective AUC' is the total of each IAUC for each toxin from time point zero to that time at which the animals began to recover from the anorectic effects.

Table 7. Comparison of percent feed refusal for type A trichothecenes as determined from incremental area under the curve (IAUC)s

		Percent refusal per feeding period (%)	
Time Point (h)	Duration ΔT (h)	T-2	HT-2
0.5	0.5	63	86
1	0.5	28.7	34
2	1	29.3	62.4
3	1	43.8	50.6
6	3	41.8	35.3
16	10	4.2	2.1
24	8	-57.8	-71.9
48	24	-24.9	-28.1

Percent feed refusal was calculated as the difference in the amount of food consumed by animals given 2.5 mg/kg bw of each toxin by oral gavage and the control group between time points. A negative value indicates a recovery from the trichothecene toxicity.

Therefore, the AUC for DON was calculated up to 3 h post-exposure, the AUC for 3-ADON and 15-ADON was calculated up to 6 h post-exposure, and the AUC for NIV, FX, T-2, and HT-2 was calculated up to 16 h post-exposure (Table 8). Additionally, the difference in dose of the Type A and Type B trichothecenes was taken into account; AUC values for both T-2 and HT-2 were multiplied by a factor of 5 for calculation of relative potency. From these AUC values a relative potency for each trichothecene was calculated with DON set as the reference mycotoxin. The relative potency based on the AUC shows, similar trends to those found in the BMD analysis. DON, 3-ADON, and 15-ADON have similar potency while NIV, FX, T-2, and HT-2 all have higher toxicity. However, these calculations appear to indicate that while a very small

amount of T-2 can cause a toxic response the overall anorectic effect is 4-fold lower than the potency value of the BMD would indicate. Based on the AUCs calculated for these 7 mycotoxins via oral exposure, the order of toxicity is DON < 3-ADON ≈ 15-ADON < NIV < FX << T-2 < HT-2.

Table 8. Total anorexic effects and relative potency to deoxynivalenol (DON) based on total area under the curve (AUC)

Toxin	Dose (mg/kg bw)	Duration (h)	Effective Total AUC (mg.h)	Relative Potency
DON	2.5	3	1.7	1
3-ADON	2.5	6	3.1	2
15-ADON	2.5	6	3.2	2
NIV	2.5	16	12	7
FX	2.5	16	15.8	10
T-2	0.5	16	10.2	31
HT-2	0.5	16	11.3	34

DON = deoxynivalenol; ADON = acetyldeoxynivalenol; NIV = nivalenol; FX = fusarenon-X; T-2 = T-2 toxin; HT-2 = HT-2 toxin.

4. Discussion

Addressing the toxicity, risk assessment, and regulation of complex mixtures of toxicants is difficult, but development of TEFs for compounds with similar modes of action and that commonly occur together is the standard for polyhalogenated aromatic hydrocarbons. The TEF model uses individual toxicity assays to make comparative measurements of toxicity called relative effect potency. The relative potencies are used to create the scaling factor known as the TEF (Van den Berg *et al.*, 2006b). Trichothecene mycotoxins typically occur in foods as

mixtures creating the potential for each to contribute to toxicity. In an effort to streamline risk assessment of such complex mixtures, we have determined the relative potencies of seven trichothecenes most commonly present in grain-based foods. The relative potencies were calculated based on a common toxicological endpoint of anorexia in a mouse model using DON as the reference trichothecene mycotoxin. Relative potencies were characterized with two separate parameters from the anorexia studies: (1) calculated BMD values; and (2) duration of anorectic effect and IAUC.

The limited number of dose groups available for our analysis made use of BMD values more desirable to calculate relative potency as opposed to NOAEL or LOAEL values. The BMD falls within the much wider range defined by the NOAEL and LOAEL allowing for more precise values and not limiting the analysis to the specific doses given in the different treatment groups. The BMD values indicate that the toxicity of T-2 is much higher than the rest of the trichothecenes following oral exposure followed by FX >> HT-2 > NIV > 15-ADON ≈ 3-ADON ≈ DON. The results from the BMD analysis showed that T-2 is about 124 times more potent than DON after oral administration and that it is also 20 times more potent than HT-2. A major caveat to this approach, however, is that the BMD values from one time point (i.e. 2 h) do not fully encompass the effect of each trichothecene on anorexia due to the differences in metabolism and thus, onset and duration of effect. Therefore, we determined that time-course analysis utilizing IAUC values to increase our accuracy in determining relative potencies.

To our knowledge, this is the first time duration of toxic effect and IAUC values have been utilized to calculate relative potencies. The endpoint of toxicological effect, anorexia, and the differences in metabolism and onset of effect of the trichothecenes necessitated inclusion of duration of effect as an important component of overall toxicity. FX, NIV, T-2, and HT-2 toxin

caused the longest duration of anorectic effects, lasting at least 10 h longer than the effects of DON, 3-ADON, and 15-ADON. The IAUC calculations were utilized to determine relative potencies of each trichothecene in relation to DON for the duration of effect. Based on this variable the toxicities are as follows: HT-2 > T-2 >> FX > NIV > 15-ADON ≈ 3-ADON > DON. Based on IAUC values, T-2 and HT-2 cause a similar overall decrease in feed intake over 16 h. FX and NIV result in the slowest recovery from anorexia, thus making them have an overall higher decrease in feed intake compared with animals treated with the other Type B trichothecenes. Similar to the results from the BMD analysis, DON, 3-ADON, and 15-ADON all have the least effect on feed consumption and are relatively similar in duration of toxic effects.

Through BMD and IAUC analysis, we were able to calculate the potency of each trichothecene relative to DON. It is important to note that while these potencies could be useful in determination of toxicity of complex mixtures of these mycotoxins and may be helpful in risk assessment and regulatory practice, there are limitations to their use as TEFs. The TEF approach for risk assessment makes multiple assumptions about the chemicals in question including; the effects of individual chemicals in a mixture are essentially additive at submaximal levels of exposure, the dose-response curves should be parallel, the individual compounds all act through the same biologic or toxic pathways, and the manifestations of all congeners must be identical over a relevant range of doses (Nagao *et al.*, 1993; Safe, 1998). The trichothecenes do not strictly adhere to each of these assumptions. For instance, while there has been abundant work on the mechanism of action for the trichothecenes, the majority of the work is focused on DON exclusively. Differences in time of onset and recovery from anorectic effects studied would indicate that the trichothecenes may act through various metabolic and toxicological pathways. Another limitation is that the feeding experiments measured feed intake 2, 2-16, and 16 h after

initial consumption; so we did not have data for a shorter period (below 2 h). In the future, a way to avoid biases in one-time point (or several-time point) studies is to compare the effects at T_{max} for each trichothecene. A final limitation is that for the trichothecenes NIV and FX, the animals did not recover from anorectic effects at the dose administered during the 16-h experimental period; hence, we could not determine from the data the actual time at which feed intake would increase again.

An understanding of the relative potencies of compounds that are known to occur in mixtures is important for making impactful and accurate risk and hazard assessments and distinguishing regulatory limits. More research needs to be conducted on the effects of these mycotoxins in mixtures to determine their true modes of action and their cumulative effect on anorexia. A more complete understanding of how these trichothecenes interact in mixtures would promote the development of TEF values to be used during risk assessment. A similar concept to establishing TEFs for the trichothecene mycotoxins should be explored further and validated through multiple animal models for future regulatory work in the food and feed industry.

CHAPTER FIVE: Modeling the emetic potencies of food-borne trichothecenes by benchmark dose methodology

Data in this chapter has been published in Male, D., Wu, W., Mitchell, N.J., Bursian, S., Pestka, J.J. and Wu, F., 2016. Modeling the emetic potencies of food-borne trichothecenes by benchmark dose methodology. Food and Chemical Toxicology 94: 178-185. DOI: http://dx.doi.org/10.1016/j.fct.2016.06.009.

Abstract

Trichothecene mycotoxins commonly co-contaminate cereal products where they cause immunosuppression, anorexia, and emesis in multiple species. Dietary exposure to such toxins often occurs in mixtures. Hence, if it were possible to determine their relative toxicities and assign toxic equivalency factors (TEFs) to each trichothecene, risk management and regulation of these mycotoxins could become more comprehensive and simple. We used a mink emesis model to compare the toxicities of deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, nivalenol, fusarenon-X, HT-2 toxin, and T-2 toxin. These toxins were administered to mink via gavage and intraperitoneal injection. The United States Environmental Protection Agency (US-EPA) benchmark dose software was used to determine benchmark doses for each trichothecene. The relative potencies of these toxins were calculated as the ratios of their benchmark doses to that of DON. Our results showed that mink were more sensitive to orally administered toxins than to toxins administered by IP. T-2 and HT-2 toxins caused the greatest emetic responses, followed by FX, and then by DON, its acetylated derivatives, and NIV. Although these results provide key information on comparative toxicities, there is still a need for more animal based studies focusing on various endpoints and combined effects of trichothecenes before TEFs can be established.

Keywords: mycotoxins, deoxynivalenol, emesis, toxic equivalency factor

1. Introduction

Trichothecene mycotoxins, produced most commonly by the fungi *Fusarium graminearum, F. culmorum,* and *F. sporotrichioides*, often contaminate common cereal grains such as wheat, barley, rye, and oats (Foroud and Eudes, 2009; Stanciu *et al.*, 2015). The most commonly occurring and regulated trichothecene is deoxynivalenol (DON), also called "vomitoxin," as it induces emesis in multiple species. The United States Food and Drug Administration (FDA) has set an industry guideline for the maximum allowable concentration of DON at 1 mg/kg (FDA, 2010). Other regulatory bodies such as the European Commission have set similar or more stringent DON standards in food (Commission, 2006).

DON is one of the trichothecenes that make up the sub-class type B trichothecenes. The type B trichothecenes are characterized by a keto group at carbon-8 of the parent epoxy-trichothecene nucleus. This group includes five associated congeners: DON, its acetylated derivatives 15-acetyldeoxynivalenol (15-ADON) and 3-acetyldeoxynivalenol (3-ADON), nivalenol (Supriya *et al.*) and its acetylated derivative fusarenon X (FX) (Fig. 7). In addition to the type B trichothecenes, there are those trichothecenes classified as type A, of which T-2 and HT-2 toxins are the most toxic. Type A trichothecenes are characterized by the presence of a hydroxyl group, ester function, or no oxygen substitution at carbon-8 (McCormick *et al.*, 2011). DON and its related trichothecene mycotoxins cause a variety of adverse effects in multiple species. These adverse effects have been reviewed in previous publications (Pestka, 2010b; Pestka, 2010c) and include emesis, nausea, anorexia, diarrhea, growth retardation, neuroendocrine effects, and disruption of the immune system. The primary clinical signs associated with exposure to DON

and related trichothecenes in human populations are nausea and vomiting. Thus, it is reasonable that these effects should be the endpoints used in risk assessment for human exposure to trichothecenes.

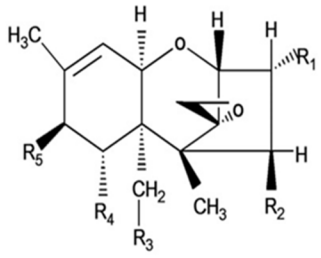
Structure	Trichothecene	Abbreviation	R1	R2	R3	R4	R5
	deoxynivalenol	DON	OH	H	OH	OH	=O
	3-acetyldeoxynivalenol	3-ADON	OAc	H	OH	OH	=O
	15-acetyldeoxynivalenol	15-ADON	OH	H	OAc	OH	=O
	nivalenol	NIV	OH	OH	OH	OH	=O
	fusarenon X	FX	OH	OAc	OH	OH	=O
	HT-2 toxin	HT-2	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
	T-2 toxin	T-2	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$

Figure 7. The general structure of trichothecenes and their various functional groups

Gastroenteritis outbreaks in the U.S. and abroad have been associated, inconclusively, with DON contamination of foods. From October 1997 to October 1998 there were 16 outbreaks of gastrointestinal illness affecting more than 1,900 school children following the ingestion of burritos from two unrelated companies (Steinberg *et al.*, 2006). The children who consumed the burritos suffered nausea, headache, abdominal cramps, vomiting, and diarrhea. Laboratory analysis did not find contamination with common bacterial strains associated with gastroenteritis, suggesting that the symptoms observed were due to a toxin contamination. Some burrito samples had detectable levels of DON, although they were below the FDA regulatory guideline of 1 mg/kg. Outbreaks in China from 1984 to 1991 were linked to moldy cereal grains. DON, as well as other trichothecenes, were verified in samples taken during this time period, and found at concentrations ranging from 2 to 50 mg/kg (Pestka and Smolinski, 2005). Several thousand

individuals from the Kashmir Valley of India were affected with gastroenteritis from consumption of foods made with moldy wheat. Samples were found to contain DON at 0.34 to 8.4 mg/kg (Bhat *et al.*, 1989). It is important to note, that although food samples may contain trichothecene levels below the FDA guideline, the threshold for human emesis is still unknown and that this threshold might also vary greatly due to differences in age, sex, diet, health status, genetic differences, etc. making it difficult to assess this value (Stadler *et al.*, 2003).

The Joint Expert Committee on Food Additives of the Food and Agriculture Organization and World Health Organization (JECFA) has determined a recommended acute reference dose (ARfD) for DON and its acetylated derivatives of 8 μg/kg bw/day. Although JECFA has not set a similar ARfD for NIV and FX, the EFSA has made a recommendation for a joint tolerable daily intake of 1.2 μg/kg bw/day (EFSA, 2013b). JECFA has established a provisional maximum tolerable daily intake (PMTDI) of 60 ng/kg bw/day for either T-2 toxin alone or a mixture of T-2 and HT-2 (JECFA, 2001b).

Although trichothecenes co-occur in numerous food commodities, a lack of robust toxicology and epidemiology studies makes it difficult to determine appropriate regulatory levels for each individual trichothecene. Additionally, risk assessment of foods with potential co-contamination is extremely difficult when there are limited studies on the toxicity of mixtures. Since there is a lack of consensus among governmental agencies on regulation of trichothecenes, assigning each trichothecene a toxic equivalency factor (Bianco *et al.*) would be desirable for risk assessment and regulatory purposes. Using such an approach, a single regulatory standard could be set for the sum total of all co-occurring trichothecenes in cereals and their products.

TEF values were first introduced in the practice of regulatory risk assessment for the polychlorinated dibenzodioxins (PCDDs), the polychlorinated biphenyls (PCBs), and the polychlorinated dibenzofurans (PCDFs) (Van den Berg *et al.*, 1998b; Van den Berg *et al.*, 2006b). The theory behind associated TEF values is that, because compounds in a particular group (e.g., dioxins) usually co-occur, are structurally similar, have similar modes of toxicity, and have additive impacts, they are each assigned an equivalency factor in comparison to the toxicity value of a reference compound. Although there is limited evidence for the additive effect of trichothecenes when consumed as mixtures, the initiation of the process to develop a similar regulatory mechanism to that of the TEF values of PCDDs, PCBs, and PCDF is warranted and essential to future risk assessment practices for these similar, co-occurring food mycotoxins.

Previously, the relative anorectic potencies of the above-mentioned trichothecenes were calculated by Male et al. (2015), using raw data collected from a mouse anorexia study (Wu *et al.*, 2012a; Wu *et al.*, 2012b). In that analysis, two methods were used to rank the potency of each trichothecene relative to DON based on feed refusal: 1) benchmark dose (BMD) analysis and 2) incremental area under the curve (IAUC). The mouse model cannot be used to study emetic responses to trichothecenes, because mice are incapable of vomiting. Therefore, the present study applied the same principles, with a new set of data from a mink model to utilize emesis as the outcome variable. Currently, the BMD is used by regulatory bodies worldwide as the "point of departure" from which to calculate tolerable daily intakes or reference doses for humans. The BMD methodology allows for use of animal data with limited number of dose groups and small n values. Here, DON, 3-ADON, 15-ADON, NIV, FX, HT-2, and T-2 toxins were ranked by potency to induce emesis, based on their individual BMD values following oral gavage and intraperitoneal (IP) dosing in mink. Relative potencies were assigned to each toxin in

relation to DON. This work is an important initial step in developing a uniform risk assessment strategy for complex mixtures of trichothecenes.

2. Materials and Methods

2.1 Mink feeding and emesis trials

The raw emesis data used in this analysis were collected from prior mink experiments. The experimental design and procedures, used in the emesis trials (Fig. 8), were previously described by Wu et al. (2012a). Briefly, DON, 3-ADON, 15-ADON, NIV, FX, HT-2, and T-2 toxins with purity of >98% were either given by gavage or IP dosing. A total of 60 standard dark, female mink between 12 and 24 months of age were obtained from the Michigan State University Experimental Fur Farm. The animals were bred and housed in accordance with the 2010 Fur Commission guidelines. Animal treatment was in accordance with the NIH guidelines and the research was approved by the Michigan State University Institutional Animal Care and Use Committee (MSU-IACUC). All animals were acclimated for 1 week, and then fasted for 24 h before initiating the experiment. To maintain a constant volume of gastric constituents in the experimental animals, water was provided *ad libitum*.

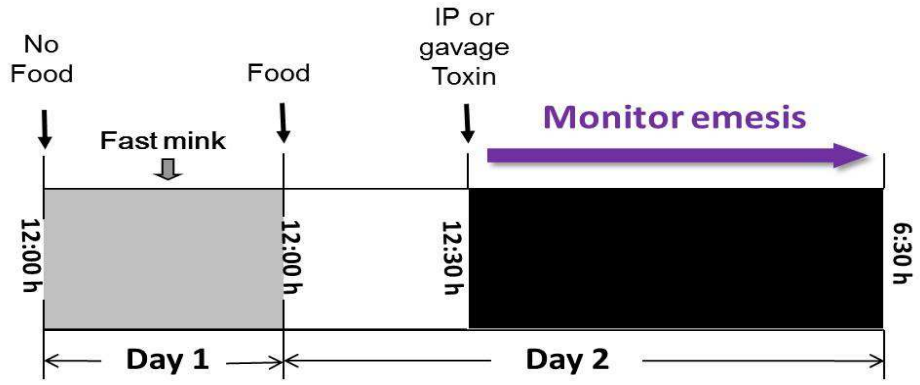


Figure 8. Experimental design for IP or gavage dosing emesis study (Wu *et al.*, 2012a)

For IP dosing studies (Fig. 8), mink were given 50 g of feed after 24 h of fasting. Thirty minutes after feeding, animals were given either phosphate buffered saline or doses of toxins in a volume equivalent to 1 ml/kg bw/day via IP injection. The animals were then monitored for emesis for 6 h. The latency to emesis, incidence of emesis, duration of emesis, and number of emetic events including retching and vomiting were recorded (Table 9). For gavage dosing studies (Fig. 8), 50 g of feed was provided to mink after 24 h of fasting. Thirty minutes following feeding, mink were given either saline or toxin in a volume equivalent to of 1 ml/kg bw/day via gavage. The animals were then monitored for emesis for 3 h and the data were recorded (Table 10).

Table 9. Results for the emetic responses of mink to trichothecenes administered by IP injection

Toxin	Doses (mg/kg bw)	Incidence	Latency (min)	Emesis duration (min)	Number of emetic events		
					Retching	Vomiting	Total
HT-2	0	0⁄4	-	-	-	-	-
	0.001	0⁄4	-	-	-	-	-
	0.01	0⁄4	-	-	-	-	-
	0.05	1⁄4	27.5 ± 0	70.5 ± 0	19.5 ± 19.5	2.8 ± 2.8	22.3 ± 22.3
	0.25	4⁄4	23.8 ± 1.6	242.1 ± 6.6	210 ± 21.5	27.8 ± 2	237.8 ± 22.2
T-2	0	0⁄4	-	-	-	-	-
	0.001	0⁄4	-	-	-	-	-
	0.01	0⁄4	-	-	-	-	-
	0.05	1⁄4	30 ± 0	55 ± 0	17.5 ± 17.5	2.3 ± 2.3	19.8 ± 19.8
	0.25	4⁄4	27 ± 3.2	206.5 ± 7.2	161.3 ± 15.1	22.5 ± 2.3	183.8 ± 17.2
3-ADON	0	0/3	-	-	0 ± 0	0 ± 0	0 ± 0
	0.1	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.2	3/6	36 ± 14	3 ± 2	7 ± 4	2 ± 1	9 ± 5
	0.3	5/6	48 ± 9	26 ± 7	28 ± 8	6 ± 2	34 ± 9
	0.4	6/6	19 ± 4	54 ± 2	81 ± 10	16 ± 1	97 ± 11

Table 9 (cont'd)

15-ADON	0	0/5	-	-	0 ± 0	0 ± 0	0 ± 0
	0.1	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.25	5/6	12 ± 2	3 ± 1	26 ± 8	4 ± 1	31 ± 9
	0.5	5/6	11 ± 1	24 ± 8	38 ± 9	7 ± 2	45 ± 11
	1	6/6	8 ± 1	32 ± 5	58 ± 5	13 ± 3	71 ± 7
DON	0	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.025	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.05	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.1	3/6	14 ± 2	6 ± 3	14 ± 8	4 ± 2	17 ± 9
	0.25	6/6	10 ± 2	38 ± 5	94 ± 13	16 ± 1	110 ± 14
FX	0	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.025	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.05	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.1	5/6	63 ± 19	22 ± 4	18 ± 5	5 ± 1	23 ± 6
	0.25	6/6	20 ± 4	91 ± 22	136 ± 8	26 ± 3	162 ±10
NIV	0	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.01	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.05	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.1	6/6	83 ± 8	56 ± 23	33 ± 7	9 ± 2	42 ± 9
	0.25	6/6	29 ± 5	158 ± 12	132 ± 20	30 ± 2	162 ±20

HT-2 = HT-2 toxin; T-2 = T-2 toxin; DON = deoxynivalenol; 3-ADON = 3-acetyldeoxynivalenol; 15-ADON = 15-acetyldeoxynivalenol; NIV = nivalenol; FX = fusarenon-X. N=4 for Type A and N=6 for Type B trichothecenes. The data presented were generated by studies conducted by Wu et al. (2012a) and reported in an earlier publication.

Table 10. Results for the emetic responses of mink to trichothecenes administered by gavage method

Toxin	Doses (mg/kg bw)	Incidence	Latency (min)	Emesis duration (min)	Number of emetic events		
					Retching	Vomiting	Total
HT-2	0	0⁄4	-	-	-	-	-
	0.005	0⁄4	-	-	-	-	-
	0.05	3⁄4	27 ± 7.1	49.7 ± 11.2	60.3 ± 24.4	7.3 ± 2.9	67.5 ± 27.3
	0.25	4⁄4	21.6 ± 3.8	120.6 ± 21.2	157.3 ± 24.2	17.8 ± 2.4	175 ± 26.4
	0.5	4⁄4	19.1 ± 1.8	200.6 ± 18.1	222.3 ± 28.5	23 ± 3.2	245.3 ± 31.2
T-2	0	0⁄4	-	-	-	-	-
	0.005	0⁄4	-	-	-	-	-
	0.05	3⁄4	22.5 ± 2.6	52.3± 15.4	66.3±22.6	8±3.1	74.3 ± 25.5
	0.25	4⁄4	19.5 ± 3.3	108 ± 11.1	130 ± 16.9	17.3 ± 1.3	147.3 ± 18.1
	0.5	4⁄4	13.1 ± 3.2	170.9 ± 22.1	191.3 ± 50.4	21 ± 2.6	212.3 ± 52.7
3-ADON	0	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.05	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.25	1/6	62 ± 0	1 ± 0	2 ± 2	1 ± 1	3 ± 3
	0.5	5/6	53 ± 9	12 ± 9	16 ± 8	4 ± 1	20 ± 9
	1	6/6	19 ± 4	44 ± 9	65 ± 11	14 ± 2	79 ± 13
15-ADON	0	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.01	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.1	5/6	20 ± 4	1 ± 1	9 ± 3	2 ± 1	11 ± 4
	0.5	6/6	18 ± 3	3 ± 1	24 ± 5	6 ± 1	30 ± 5
	1	6/6	17 ± 4	14 ± 7	26 ± 5	6 ± 1	32 ± 6
DON	0	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.01	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.05	5/6	15 ± 2	1 ± 1	12 ± 4	3 ± 1	15 ± 5
	0.25	6/6	17 ± 2	6 ± 1	44 ± 13	8 ± 1	52 ± 15

Table 10 (cont'd)

DON	0.5	6/6	11 ± 2	14 ± 3	62 ± 9	9 ± 1	71 ± 10
FX	0	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.01	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.05	4/6	23 ± 2	13 ± 10	8 ± 3	2 ± 1	10 ± 4
	0.25	6/6	36 ± 10	34 ± 11	38 ± 5	11 ± 2	49 ± 7
	0.5	6/6	15 ± 3	63 ± 21	45 ± 4	13 ± 2	58 ± 4
NIV	0	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.05	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.1	0/6	-	-	0 ± 0	0 ± 0	0 ± 0
	0.25	4/6	31 ± 3	15 ± 10	21 ± 14	4 ± 2	25 ± 16
	0.5	6/6	29 ± 4	32 ± 10	55 ± 14	9 ± 2	64 ± 15

HT-2 = HT-2 toxin; T-2 = T-2 toxin; DON = deoxynivalenol; 3-ADON = 3-acetyldeoxynivalenol; 15-ADON = 15-acetyldeoxynivalenol; NIV = nivalenol; FX = fusarenon-X. N = 4 for Type A and N = 6 for Type B trichothecenes. The data presented were generated by studies conducted by Wu et al. (2012a) and reported in an earlier publication.

2.2 Benchmark dose modeling and calculation of relative emetic potency

The United States Environmental Protection Agency (EPA) benchmark dose software version 2.6.0.1 (BMDS 2.6.0.1) was used to model and calculate the BMD of each mycotoxin. The raw data, including the doses, number of animals per dose group, and number of animals that vomited were entered into an in-built excel wizard (Version 1.10) for dichotomous data. The variable from Wu et al. (2012a) utilized to calculate the BMD values were "incidence" for IP (Table 9) and gavage (Table 10) at the various doses. The BMD was determined as the point of departure at a benchmark response of 10% (BMR_{10}). We calculated the averaged BMD_{10} values and 95% confidence interval for the incidence of emesis for each trichothecene from results of parametric bootstrapping, using 500 iterations. The $BMDL_{10}$ was derived from the lower one-sided 95% confidence interval of the distribution for each dose group (Crump, 1995).

Dose-response data for each toxin were fit to several built-in mathematical models in BMDS, including gamma, dichotomous-Hill, logistic, log-logistic, probit, log-probit, Weibull, multistage 2˚, 3˚, and 4˚ cancer, and quantal linear models. The model that provided the best fit for the experimental dose-response data was selected based on the ratio of BMD to BMDL (a ratio closer to 1 indicates lower uncertainty and variability), Akaike information criterion, goodness-of-fit *p* values, scaled residuals for dose groups near the BMD calculated, visual inspection of graphical outputs (fit of curve to dose-response points) and whether there were any BMDS model warnings (EPA, 2012). The BMD analysis procedure was done for all the trichothecenes studied, and their relative emetic potencies were calculated as the ratio of the BMD of DON to the BMD of each of the other trichothecenes. The procedure for calculating relative potency was described in our previous publication (Male *et al.*, 2015).

2.3 Fixed dose comparison of emetic events

Additionally, we summarized and compared the number of emetic events ("total" under "number of emetic events" in Table 10) following oral administration of 0.5 mg/kg bw of each of the toxins. This dose was selected since it was the common dose in all investigations. These values, "total number of emetic events", for all the trichothecenes were divided by that of DON to determine their relative potencies.

3. Results

3.1 Intraperitoneal dosing experiments

The IP dosing BMDs for the type B trichothecenes were 73, 108, 141, 63, and 60 μg/kg bw for DON, 15-ADON, 3-ADON, NIV, and FX, respectively. The BMDs for HT-2 and T-2 toxins administered by IP injection were both equal to 31 μg/kg bw and identical (Table 11), suggesting

that they were equally emetic to mink through this exposure route. The BMD, or minimum amount of toxin required to induce vomiting in mink by IP injection, was greatest for 3-ADON (i.e., 3-ADON was the least emetic); followed in decreasing order by 15-ADON, DON, NIV, FX, and HT-2 or T-2 toxins. The relative potencies of the trichothecenes were calculated as the ratio of the BMD of DON to that of each of the other toxins. Thus, the calculated rank order of the IP exposure emetic potencies was: HT-2/T-2, followed in decreasing order by FX, NIV, DON, 15-ADON, and 3-ADON (Table 11). Moreover, the emetic potency of DON was about 1.7 times higher than that of either 3-ADON or 15-ADON. Generally, the BMDs of the group of DON, 3-ADON and 15-ADON were higher than the BMDs of NIV and its acetylated derivative FX, implying that DON and its derivatives had a lower relative emetic potency than either NIV or FX. The order of potency based on IP administration was: HT-2 ≈ T-2 > FX > NIV > DON > 15-ADON > 3-ADON.

Table 11. The emetic benchmark doses and relative potencies of type-A and type-B trichothecenes administered to mink using gavage (oral) and IP dosing methods

Type	Trichothecene	IP BMD (μg/kg bw)	IP Relative potency	Gavage BMD (μg/kg bw)	Gavage Relative potency [2]
A	HT-2	31	2.38	14	1.73
A	T-2	31	2.38	14	1.73
B	3-ADON	141	0.52	198	0.12
B	15-ADON	108	0.67	40	0.60
B	DON	73	1.00	24	1.00
B	FX	60	1.22	23	1.04
B	NIV	63	1.16	141	0.17

DON = deoxynivalenol; 3-ADON = 3-acetyldeoxynivalenol; 15-ADON = 15-acetyldeoxynivalenol; NIV = nivalenol; FX = fusarenon-X; HT-2 = HT-2 toxin; T-2 = T-2 toxin. Relative emetic potencies are the ratio of the BMD of DON to the BMD of each of the other trichothecenes studied.

3.2 Gavage dosing experiments

The oral (gavage) BMDs for the type B trichothecenes were 24, 40, 198, 141, and 23 μg/kg bw for DON, 15-ADON, 3-ADON, NIV, and FX, respectively. The rank order of the calculated emetic potency of each toxin was FX, DON, 15-ADON, NIV, and 3-ADON, respectively; with FX being the most toxic type B trichothecene and 3-ADON the least toxic (Table 11). The oral exposure emetic BMD of both T-2 and HT-2 toxins was 14 μg/kg bw; it was lower than the BMDs of all type B trichothecenes, indicating a greater toxicity of T-2 and HT-2 toxins. Via oral exposure, DON was 8.3 and 1.7 times more potent than 3-ADON and 15-ADON, respectively. Unlike IP dosing where the BMD of 3-ADON was slightly higher than that for 15-ADON (141 and 108 μg/kg bw respectively), for oral administration, the BMD for 3-ADON was 5 times the value for 15-ADON (198 and 40 μg/kg bw respectively). The oral emetic potencies of NIV and FX relative to DON were 0.17 and 1.04 respectively, indicating that the potential for FX to induce emesis in mink was 6 times that of NIV. The order of potency based on gavage was: HT-2 ≈ T-2 > FX > DON > 15-ADON > NIV > 3-ADON.

3.3 Comparing emetic potency for IP and gavage routes of exposure

The BMD analysis also revealed interesting findings about the two routes of exposure. Generally, the emetic potency of the trichothecenes was higher via the gavage route than via IP injection. With the exception of NIV and 3-ADON whose IP injection BMD values were lower, the calculated values for IP exposure were higher for T-2, HT-2, FX, DON, and 15-ADON than those calculated when the trichothecenes were administered by gavage. For HT-2 and T-2 toxins, the BMDs were 31 and 14 μg/kg bw for IP and gavage dosing, respectively. 3-ADON had the largest BMD values for either exposure routes, thus the lowest potency of the seven common dietary trichothecenes. The findings further show that irrespective of exposure route, DON is

more toxic than both 15-ADON and 3-ADON. The oral emetic potency of FX is 6 times higher than that of NIV. However, there was no difference in toxicity between the two toxins when introduced to mink by IP injection, because their emetic potencies were the same. The potency of NIV relative to DON is 1.22 when given by IP dosing; but when administered orally, DON is 6 times more potent than NIV.

3.4 Comparing the emetic events from oral exposure to a fixed dose of trichothecenes

The number of emetic responses to a fixed oral dose - 0.5 mg/kg bw- of type A and type B trichothecenes is summarized in Table 12 (Wu *et al.*, 2012a). Type A trichothecenes caused the most incidents of emesis as compared to their type B counterparts. The sum of the frequencies of retching and vomiting were not significantly different for HT-2 and T-2 toxins. Among the type B trichothecenes, DON produced the highest number of responses followed in decreasing order by NIV, FX, 15-ADON, and 3-ADON. The number of incidents caused by FX, DON and NIV were not significantly different. Similarly, the sum of emetic events caused by 15-ADON and 3-ADON did not differ significantly. The total number of emetic events produced by FX, DON, or NIV was significantly higher than the totals for either 15-ADON or 3-ADON (Table 12). In summary, the rank order of total emetic events produced by each toxin when a uniform dose equal to 0.5 mg/kg bw was administered was HT-2 ≈ T-2 > DON ≈ FX ≈ NIV > 15-ADON ≈ 3-ADON.

Table 12. The number of emetic responses by mink administered a fixed oral dose (0.5 mg/kg bw) of type-A and type-B trichothecenes

		Number of emetic events		
Type	Trichothecene	Retching	Vomiting	Total
A	HT-2	222	23	245 ± 32^{a}
A	T-2	191	21	212 ± 53^{a}
B	3-ADON	16	4	20 ± 9^{c}
B	15-ADON	24	6	30 ± 5^{c}
B	DON	62	9	71 ± 10^{b}
B	FX	45	13	58 ± 4^{b}
B	NIV	55	9	64 ± 15^{b}

DON (deoxynivalenol), 3-ADON (3-acetyldeoxynivalenol), 15-ADON (15-acetyldeoxynivalenol), NIV (nivalenol), FX (fusarenon X), HT-2 (HT-2 toxin), T-2 (T-2 toxin). The frequency of retching and vomiting were counted as discrete events. The total number of emetic events was calculated as the sum of retching and vomiting. The 0.5 mg/kg oral dietary dose was the common dose included in all animal sub-studies conducted by Wu et al. (2012a) when investigating the emetic responses of mink to a range of doses of trichothecenes. Total emetic events recorded as Means ± SD. Different letters superscripted under "Total" indicates those groups are significantly different ($p \leq 0.05$).

4. Discussion

As more mycotoxins are being regulated in foods worldwide, challenges arise when setting appropriate standards for each. Because the trichothecene mycotoxins are produced by the same groups of fungi, co-contaminate the same cereal crops, and cause largely the same adverse health effects, regulation of these mycotoxins can be simplified by setting TEFs relative to a single toxin. This is the first study to compare the emetic potencies of trichothecenes, using the BMD

method, to find appropriate points of departure for risk assessment purposes and to develop relative potencies for each toxin relative to DON.

In the case of emesis from trichothecene exposure, we found that the animals were more sensitive to gavage than to IP administration. This is because the vomiting center in the brain is stimulated when either the receptors in the periphery (gastrointestinal tract) or in the blood are activated by the presence of toxins (Horn, 2008; Prelusky and Trenholm, 1993). Studies have shown that oral exposure to trichothecenes causes gastroenteritis (Pestka, 2010c), which activates the enterochromaffin cells in the epithelium to release 5-hydroxytryptamine (5-HT) or serotonin. The 5-HT neurotransmitter sends signals to the brain vomiting center to induce vomiting (Prelusky and Trenholm, 1993). Likewise, introduction of toxins into the blood by IP dosing or absorption from the gut into the systemic circulation can stimulate the vomiting center via direct activation of 5-HT_3 receptors in the chemoreceptor trigger zone (CTZ) in the brain (Becker, 2010; Dietrich *et al.*, 2015; Horn, 2008; Kovac, 2016; Lang, 1999; Prelusky and Trenholm, 1993). This suggests that oral exposure could result in double stimulation of the vomiting center i.e. via 5-HT receptors in the gut before absorption, and via 5-HT_3 receptors in the CTZ during systemic circulation, eliciting a higher emetic response than via IP exposure. The IP route induces vomiting via only the CTZ, which may explain the lower emetic potency as compared to the oral route. Human consumption of trichothecene contaminated foods is an oral exposure making the relative potencies of the gavage model more appropriate for human risk assessment.

The oral potency decreased from DON to 15-ADON to 3-ADON, respectively. This result is consistent with other studies that demonstrated that acetylation of DON at carbon-3 (C-3) to 3-ADON decreases toxicity (Alexander *et al.*, 1999; Kimura *et al.*, 1998; McCormick, 2013; Zhou

et al., 2008). In contrast to a number of previous studies that indicated that acetylation of DON at C-15 to 15-ADON increases cytotoxicity (Desjardins *et al.*, 2007), this study showed that the emetic potency of DON was greater than 15-ADON. The low toxicity of 15-ADON *in vivo* could be partly due to deacetylation to DON in the intestine prior to absorption (Veršilovskis *et al.*, 2012) and in part, attributed to faster rates of clearance as compared to DON (Broekaert *et al.*, 2015). In pigs, the rapid and nearly complete pre-systemic hydrolysis (99%) of 15-ADON to DON was observed. However, the rates of clearance were in increasing order: 3-ADON > 15-ADON > DON (Broekaert *et al.*, 2015). Although both 3-ADON and 15-ADON ultimately undergo hydrolysis in the intestine before absorption, each toxin (DON, 3-ADON, and 15-ADON) exerts a different toxicity on the local tissue (Broekaert *et al.*, 2015). For example, Pinton et al. (2012) used *in vitro, ex vivo* and *in vivo* studies to compare the effects of DON, 3-ADON and 15-ADON on the barrier function of intestinal cells and activation of MAPK. The study revealed that 15-ADON caused more severe effects including histological lesions, activation of MAPK and decreased expression of tight junction proteins than DON and 3-ADON (Pinton *et al.*, 2012b).

IP dosing results show that the BMD for NIV and FX are similar i.e. 60 and 63 µg/kg bw respectively. The similar emetic potency could be explained by reports that FX is metabolized to NIV in the liver and kidney after absorption, suggesting that NIV mediates FX's *in vivo* toxicity (Poapolathep *et al.*, 2003). Our results further indicated that the oral exposure potency of FX was equivalent to that of DON, but 6 times higher than that of NIV. This observation is contrary to previous *in vitro* studies that have reported higher toxicities for NIV and FX as compared to DON (Eriksen *et al.*, 2004; Wu *et al.*, 2013d). The higher rate of absorption for DON (Avantaggiato *et al.*, 2004; Kongkapan *et al.*, 2016; Pralatnet *et al.*, 2015) could counterbalance

the differences in emetic potency. The higher toxicity of FX as compared to NIV is consistent with an earlier finding that FX was more efficiently absorbed than NIV (Poapolathep *et al.*, 2003). The limited absorption of NIV following oral gavage could have reduced its emetic potency, because a large amount of NIV might have passed through the gastrointestinal tract without being absorbed (Poapolathep *et al.*, 2003). Studies have shown that the presence of certain functional groups at C4 of type B trichothecenes also influences their toxicity. For instance, at the C4 position, the toxicity of the groups is in the order: acetyl > hydroxyl > hydrogen groups (Zhou *et al.*, 2008). Additionally, studies using *A. thaliana* leaf model showed that acetylation at the C4 position increased cytotoxicity of trichothecenes (Desjardins *et al.*, 2007). This would indicate that the change in the acetyl group of FX to a hydroxyl group in NIV would decrease FX's toxicity *in vivo*, making it more toxicologically similar to DON.

Type A trichothecenes T-2 and HT-2 had the highest emetic potencies among all the toxins investigated. Although some studies have demonstrated that the toxicity of T-2 is greater than that of HT-2 toxin (Königs *et al.*, 2009; Visconti *et al.*, 1991), their emetic potencies were the same in the mink model. One possible reason is that T-2 toxin is rapidly bio-transformed to HT-2 toxin following oral exposure (Sintov *et al.*, 1986; Wu *et al.*, 2013d; Zhou *et al.*, 2008). It is then rapidly absorbed as a mixture of T-2 and HT-2, suggesting that the observed emetic events in mink are a consequence of the absorbed HT-2 (Canady *et al.*, 2001b; Conrady-Lorck *et al.*, 1988; Muro-Cach *et al.*, 2004).

The analysis of relative potencies conducted in this work and our previous publication (Male *et al.*, 2015) are important aspects to developing a methodology to assess risk of food commodities with mixtures of these trichothecenes. It is important to analyze the differences in toxicity of each trichothecene in relation to exposure route and toxic endpoint. Here we have provided

potency evaluations for emesis through two different types of data, BMD analysis of emetic incidence and comparison of total number of emetic events. In general the ranking of potency based on these two separate data sets were similar. However, it is interesting to note that although the mink requires a significantly higher dose of NIV for initiation of an emetic response compared with DON and FX, they all induce a similar number of emetic events. Additionally, a significantly lower dose of 15-ADON compared with NIV and 3-ADON will induce emesis; however 15-ADON and 3-ADON given at a common dose will induce the same number of emetic events, which is less than the number of events induced by NIV. Male et al. (2015) observed a relative potency ranking of the trichothecenes of T-2 >> FX > HT-2 > NIV > DON ≈ 3-ADON ≈15-ADON based on anorexia as an endpoint. This endpoint demonstrates that the potency of DON on emesis is significantly higher than its potency in regards to anorexia. When emesis is the observed endpoint DON acts more like FX than 15-ADON and 3-ADON. These factors need to be considered and the appropriate endpoint determined for future work.

We propose the use of the TEF approach in assessing the human health risk due to trichothecenes exposure in food. Although information about the mechanisms of action of some trichothecenes is incomplete since most available studies have focused on DON (Escrivá *et al.*, 2015; Sobrova *et al.*, 2010), the molecular structures of trichothecenes are similar enough to suggest similar mechanisms. Our data on emetic effects of each trichothecene in a mink model allow us to compare their toxicities through calculation of emetic potencies relative to DON. Another limitation to the TEF approach for trichothecenes is that very few studies have evaluated their potential interactions and consequent effects in animals and yet we know that these toxins typically co-occur in food (Bertuzzi *et al.*, 2014b; Desjardins *et al.*, 2007; Ibáñez-Vea *et al.*, 2012; Pronk *et al.*, 2002; Šegvić Klarić, 2012). Although a recent investigation reported some

synergistic toxic effects in vitro (Alassane-Kpembi *et al.*, 2014), these are inconclusive since there is still uncertainty over the nature of interactions in vivo. Therefore, more research on emetic responses induced by mixtures of these mycotoxins using animal models is needed to inform risk assessment.

Taken together, the results of this study demonstrate that assigning of TEFs for trichothecenes that co-occur in food is possible. As mentioned earlier, for the method to be valid, the emetic effects of the compounds need to be additive (Van den Berg *et al.*, 1998b). Therefore, proper understanding of the relative potencies of mycotoxins that occur in mixtures is important for estimation of their overall toxicity.

CHAPTER SIX: Conclusions and future directions

Despite concerted global efforts to reduce dietary exposure to foodborne mycotoxins, many countries especially in Sub-Saharan Africa, still carry a disproportionate health and economic burden of these toxins. *Aspergillus spp* molds, especially AFB_1 remain a major problem in the tropics due to the warm and humid climates that promote its growth as well as failure to effectively regulate mycotoxins. Similarly, *Fusarium* molds still pose a significant health burden in cooler temperate regions where they co-contaminate food and produce a cocktail of trichothecene mycotoxins. In this dissertation, we investigated the level of exposure to AFB_1 among Ugandan children and discussed possible ways to sustainably reduce exposure to food borne toxins among low income communities. We also presented approaches to more precisely assess the risk due to mixtures of trichothecenes. The work presented in each chapter is summarized below.

In chapter 2, we assessed the health burden due to exposure to aflatoxins among Ugandan children aged between 1 and 5 years from different geographical locations as well as residence types. Our study found that, Ugandan children are ubiquitously exposed to harmful levels of aflatoxins. The level of exposure varies significantly across regions but there was no heterogeneity in aflatoxin exposure among children from various household residence types, age groups, as well as between males and females. We also found that the extent of exposure among Ugandan children poses a significant risk to normal child growth and optimum immune system function. However, our study did not generate sufficient evidence to suggest whether or not their level of exposure posed a significantly increased risk of HCC presently or later in life. While the AFB_1-lysine adduct is a well validated estimator of internal dose and chronic exposure, future

studies should consider measuring the AFB_1-N^7-guanine biomarker too since its concentration is directly associated with the p53 mutation which is risk factor for HCC.

In chapter 3, we discussed the benefits of dietary diversity as a cost effective and sustainable approach to reduce exposure to foodborne mycotoxins. We found that, programs focusing on improvement of crop biodiversity in resource-poor countries have the dual benefit of improving nutrition among communities, as well as decreasing exposure to foodborne toxins due to partial substitution of the potentially contaminated staple foods with healthier alternatives. Additionally, consumption of a nutritionally diverse diet could increase the intake of food constituents that counteract the adverse effects of toxins in the body. Dietary diversity can be easily incorporated into already existing food traditions since it does not advocate for complete abandonment of existing staples. Therefore, dietary diversity must be considered alongside other agricultural and nutritional strategies to control exposure to mycotoxins. We also recommend that, to effectively improve human health through dietary diversity, a multi-sectoral approach involving agriculture, nutrition, toxicology, extension, marketing, education, and policy development experts is required to develop sustainable interventions.

In chapter 4, we analyzed data for type A and type B trichothecenes from a murine food refusal bioassay using the US-EPA BMD method and IAUC method, and calculated the oral anorectic potency of each trichothecene relative to that of DON. Based on the BMD method, the relative anorectic potencies of the 7 most common food borne trichothecenes when administered via the oral exposure route were: DON ≈ 3-ADON ≈ 15-ADON < NIV < FUS-X < HT-2 << T-2. The ranking of oral anorectic potencies for the same toxins determined by the IAUC methodology was: DON < 3-ADON ≈ 15-ADON < NIV < FUS-X << T-2 < HT-2. Although there are general limitations to the use of TEFs on trichothecenes including the variations in the *Fusarium spp*

profile in foods with climate and seasonal changes as well as limited information on interactions among trichothecenes, our study demonstrated that the TEF concept is feasible for foodborne trichothecenes which typically co-occur in foodstuffs, and has the potential to improve risk assessment practice and food safety regulation.

In chapter 5, we used the benchmark dose modeling approach to calculate and compare the relative emetic potencies of trichothecenes in mink. Similar to the murine study in chapter 4, our goal was to illustrate how TEFs could be used to develop a uniform assessment strategy for complex mixtures of trichothecenes. We used data from previous mink feeding trials to calculate the BMD for each toxin following both IP and gavage dosing. The order of potency based on IP administration was: HT-2 ≈ T-2 > FX > NIV > DON > 15-ADON > 3-ADON. For oral/gavage exposure, the rank order of potency was: HT-2 ≈ T-2 > FX > DON > 15-ADON > NIV > 3-ADON. Generally, the emetic potency of the trichothecenes was higher via the gavage route than via IP injection. Since human consumption of trichothecene contaminated foods is an oral exposure, the relative potencies of the gavage model are more appropriate for human risk assessment than those obtained via IP dosing.

Based on the BMD values from our studies, the emetic response in the mink model provide a more conservative and more protective endpoint than feed refusal or anorectic response for regulation of human risk to trichothecenes. Taken together, the results obtained from the mouse anorexia (chapter 4) and mink emesis (chapter 5) studies demonstrate that assigning of TEFs for trichothecenes that co-occur in food is possible and could improve risk assessment.

Based on the findings in this dissertation, future research and policy initiatives should consider the following measures in order to reduce exposure and to improve the efficacy of current mycotoxin regulations:

1. The level of exposure to AFB_1 among Ugandan children needs to be explored further using both the urinary and albumin biomarker to comprehensively evaluate the impact on health as well as the risk of cancer.
2. To improve risk assessment and food safety regulation, more research needs to be conducted on combined effects of trichothecenes to determine their true modes of action using multiple animal models and toxicological endpoints. A complete understanding of how these toxins interact in mixtures would promote the development of more precise TEF values.
3. To further improve the TEF approach, future studies should also consider comparing the effects of each trichothecene at the time of maximum effect as a way to avoid biases in one-time point studies. This is due to differences in time of onset and recovery from anorectic effects of the different trichothecenes.
4. To improve dietary diversity in developing countries and among communities that rely heavily on monotonous diets, governmental programs that incentivize and make agrobiodiversity profitable to farmers and diverse foodstuffs accessible to consumers should be made a priority.
5. Food regulatory agencies worldwide should consider assigning TEFs to trichothecenes since they co-occur in food and jointly contribute towards the observed adverse effects. The overall toxicity should therefore include the contribution of each toxin in the mixture.

APPENDICES

APPENDIX A: The association between serum levels of AFB_1 biomarkers and cytokine concentration among Ugandan children aged 1-5 years with uncomplicated malaria

Data in this appendix will be included in a manuscript: Denis Male, Nicole J. Mitchell, John E. Linz, James J. Pestka, Norbert E. Kaminski, Robert B. Crawford, Archileo N. Kaaya, Dorothy Nakimbugwe, and Felicia Wu. The Association between Serum Levels of AFB_1 Biomarkers and Cytokine Concentration among Ugandan Children Aged 1-5 Years with Uncomplicated Malaria

Abstract

Cytokines play an important role in cell to cell communication and mediate processes that lead to pathogen clearance and healing. But if uncontrolled, they cause tissue damage and immunopathology. *Plasmodium falciparum* infection is associated with production of a range of pro- and anti-inflammatory cytokines that determine the clinical outcomes of malaria. In this study, we used multi-analyte flow cytometry to determine the concentrations of cytokines in serum of 267 Ugandan children aged 1-5 years with uncomplicated malaria. The participants were categorized based on gender, residence type, region, and age-group. The Kruskal-Wallis and Mann-Whitney-Wilcoxon rank tests were used to compare mean cytokine concentrations among different patient groups whereas the Spearman's correlation analysis was done to evaluate the relationships among cytokines in both patients and the control group.

Our results showed that all samples had detectable levels of IL-6 and IL-10. Moreover, these were strongly positively correlated (rho = 0.77, $p < 0.001$). About 50% of the serum samples had detectable IL-1β, IFNγ, and TNFα while the majority had undetectable IL-4 and IL-12p70 levels. There were significant differences in the level of cytokines in serum of children from different regions. However, the immune response among uncomplicated malaria patients was not

significantly affected by gender, age group and residence type. Pearson's correlation analysis revealed that the concentrations of the different cytokines were not correlated with the level of AFB_1 adducts in serum.

Our findings suggest that identifying region specific factors that modulate immune system function is important for identifying interventions that promote long-term protection from malaria. Finally, our study reveals key cytokine production patterns among uncomplicated malaria patients that may be useful to predict clinical outcomes of infection in children.

Keywords

Cytokines, Malaria, *Plasmodium falciparum*, Inflammation, Uganda

1. Introduction

Cytokines are signaling molecules that play a role in cell to cell communication to regulate immune responses (Zhang and An, 2007). They may be classified as pro- or anti-inflammatory depending on their role in host immune response modulation (Cavaillon, 2001; Zhang and An, 2007). Pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), and interferon gamma (IFNɣ) are produced in response to stress and if uncontrolled, they may engender fever and tissue destruction, thereby exacerbating disease (Dinarello, 2000; Frosch and John, 2012). In contrast, anti-inflammatory cytokines such as interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-10 (IL-10), and interleukin-13 (IL-13) counter-regulate pro-inflammatory responses by suppressing the production of pro-inflammatory cytokines (Dinarello, 2000; Zhang and An, 2007). The factors that underlie the host response towards a particular antigen are not completely understood. Some studies suggest that during T cell activation, the type and amount of antigen, the relative balance between pro- and anti-inflammatory cytokines and co-stimulatory molecules, determine the phenotype of effector T cells formed as well as the nature of the immune response (Dinarello, 2000; Kaiko *et al.*, 2008). Other studies have highlighted the role of the host immune status, genetics, as well as geographical and social factors in determining the nature of immune response and therefore severity of diseases (Angulo and Fresno, 2002; Bartoloni and Zammarchi, 2012). Excessive inflammation has been implicated in the pathogenesis of several maladies in humans including non-alcoholic fatty liver disease, chronic obstructive pulmonary disease, and malaria (Crompton *et al.*, 2014; Dima *et al.*, 2015; Jamali *et al.*, 2016).

In malaria patients, *P. falciparum* causes the majority and the deadliest form of the disease in sub-Saharan Africa (Crompton *et al.*, 2014; Gething *et al.*, 2011). Malaria has two distinct stages

of infection i.e. the pre-erythrocytic (skin tissue and hepatocytes infection) and the erythrocytic stage (red blood cell invasion). The skin and hepatocyte infections are clinically silent (Crompton *et al.*, 2014) while the erythrocytic stage is associated with a surge in the concentration of a range of circulating cytokines (Kemp *et al.*, 2002). In a *P. falciparum* blood infection, a variety of pathogen associated molecular patterns (PAMPs) including glycosylphosphatidylinositol (GPI), hemozoin, and CpG DNA-bound motifs are exposed to the host immune cells. The host's immune cells express pathogen recognition receptors (PRRs) such as toll-like receptors (TLRs) that detect specific PAMPs of invading microorganisms resulting in an inflammatory response (Gun *et al.*, 2014; Kaiko *et al.*, 2008). Innate immune cells are activated which stimulates in production of cytokines (Frosch and John, 2012; Sinnis and Zavala, 2008). Initial inflammatory responses stimulate production of IFNɣ and TNFα which are necessary to control the multiplication of parasites (Bertolino and Bowen, 2015; Laishram *et al.*, 2012; Torre *et al.*, 2002). Studies have shown that during the pre-erythrocytic stage of infection, *P. falciparum* induces production of $CD4^{+}CD25^{+}FoxP3^{+}$ T regulatory cells that increase immune tolerance and dampen inflammation (Crompton *et al.*, 2014). Consequently, these immunomodulatory effects of *P. falciparum* often result in continued multiplication of parasites in the human host (Frosch and John, 2012). During the erythrocytic stage of infection, the malaria parasites multiply inside red blood cells, which then burst to release merozoites whose PAMPs are readily recognized by host immune cells. This process induces the production of various cytokines including IL-1β, IL-6, IL-12, IFNɣ, and TNFα and also induces fever and other clinical symptoms of malaria (Crompton *et al.*, 2014). The concentrations of these cytokines in serum of malaria patients could be used to predict fatal outcomes (Angulo and Fresno, 2002). For example, excessive production of TNFα has been reported in patients with severe cerebral

malaria. Moreover, low levels of IL-12 have been associated with more severe anemia. Fortunately, the majority of malaria cases in children are uncomplicated; only about one percent (1%) of the *P. falciparum* malaria patients develop severe disease (Angulo and Fresno, 2002; Crompton *et al.*, 2014). Although the symptoms of uncomplicated malaria are often non-specific, and subjects may not appear very ill, serious complications and progression to severe malaria may occur at any stage especially in immunocompromised individuals such as pregnant women, malnourished children, and transplant recipients where progression can be rapid (Bartoloni and Zammarchi, 2012).

Previous studies have linked ingestion of AFB_1, a common contaminant in staple foods such as maize and peanuts, to immune system dysfunction since the toxin interferes with cytokine expression and lymphocytes activation (Jiang *et al.*, 2005; Meissonnier *et al.*, 2008). Because the cytokine milieu is associated with particular disease outcomes and 99% of malaria cases are uncomplicated, it is very important to understand the kind of cytokines and demographic factors that may influence host immune responses in patients with uncomplicated malaria. To this end, we determined the concentrations of aflatoxin B_1-lysine adducts and the level of cytokines in serum of Ugandan children aged 1-5 years with uncomplicated malaria and evaluated their relationship. We also assessed the effect of various demographic factors on the immune response among the patients. Identification of the uncomplicated malaria cytokine fingerprint and role of the various factors will generate critical data necessary to improve disease management and also increase the ability of health practitioners to predict which children are at a higher risk than others, of progressing to severe malaria.

2. Materials and Methods

2.1 Study population

This cross sectional study involved 267 male and female Ugandan children between 1 and 5 years of age who were diagnosed with uncomplicated malaria. The study sites included health center III and IV categories in Central (Kampala and Buikwe districts), Western (Mbarara district), Eastern (Jinja and Iganga districts) and Northern (Gulu, Pader, and Kitgum districts) Uganda. The health centers were selected based on availability of laboratory testing equipment and trained staff. The research was approved by Michigan State University Biomedical and Health IRB (MSU-BIRB; IRB No. 15-556), Makerere University School of Health Sciences-IRB committee (MakSHS-IRB; IRB No. 2015-023) and the Uganda National Council for Science and Technology (UNCST).

2.2 Informed Consent for Patients

Written consent was obtained from parents or adult guardians on behalf of the eligible child. The research team explained the details of the research study and answered questions comprehensively before the parents were requested to sign the consent form. In addition to English, the approved consent form was translated to four local languages commonly used in Uganda including Luganda, Runyankole, Acholi, and Langi. The process took place in private rooms or in a non-coercive environment provided by the health center. The research team ensured that the children whose parents consented to participate in the study received malaria treatment before leaving the hospital and that the treatment was provided within 2 hours after diagnosis.

2.3 Patient Screening Procedure

The screening process was conducted in two steps. 1) The children were tested to confirm presence of *P. falciparum* parasites in their blood using two routine tests. These included the

rapid diagnostic test (RDT) followed by microscopic examination of freshly sampled blood for presence of live parasites using the thick film smear procedure (Bejon *et al.*, 2006). 2) Children who were confirmed positive for malaria were further screened using the following inclusion criteria; the patients were HIV negative, not exposed to anti-malarial and anti-inflammatory medicines prior to blood sampling (not yet initiated malaria treatment), and prior to their current malarial infection, the patients had not suffered from malaria for at least three months. Those who were eligible to participate in the research study were requested to speak with the researchers during which informed consent was sought.

2.4 Sample collection and handling

A single peripheral blood sample of about 5 mL was drawn by a laboratory technician using venipuncture into a serum tube containing a clot activator. The sample tubes were then kept at room temperature for 30-60 minutes to allow separation of serum from cells and clotting factors (Tuck *et al.*, 2008). Serum was aliquoted into cryovials in duplicate and immediately stored in the hospital freezers. The cryovials were transferred from the hospitals to a central collection laboratory at Makerere University and stored at -80˚C. One portion of each sample was shipped on dry ice to Michigan State University for measurement of cytokine concentration and the duplicate portion was shipped to Johns Hopkins University for aflatoxin analysis.

2.5 Determination of cytokine concentration in serum

The concentrations of IFNɣ, IL-1β, IL-4, IL-6, IL-10, IL-12 p70, and TNFα in the serum of each patient were simultaneously determined by flow cytometry. The LEGENDplex™ multi-analyte bead-based immunoassay kit (BioLegend Inc., San Diego, USA) was used. The assay was performed using 96-well V-bottom microplates according to the manufacturer's protocol. Briefly, 25 µL of serum, detection beads, and biotinylated antibodies, respectively were added to

25 µL of assay buffer in a V-bottom plate. The plates were covered completely using an adhesive plastic film and aluminum foil and then shaken at 600 rpm for 2 hours. Without washing, 25 µL of the Streptavidin-Phycoerythrin conjugate (SA-PE) was added to each well and shaken again for 30 min. The plates were centrifuged at 1000 x g for 5 min and washed in a buffer. The supernatant was then removed before re-suspending the beads in wash buffer. The concentrations of the cytokines were read on a BD FACSCanto II flow cytometer (BD Biosciences, NJ USA). The Flow Cytometry Standard (FCS) files generated were analyzed using the LEGENDplex™ Data Analysis Software (BioLegend Inc., San Diego, USA). The concentrations of the cytokines in each sample were calculated from standard curves of human cytokine standards provided in the test kit. The minimum detectable concentration of cytokines in patients' serum was: IFNɤ, 2.9 pg/mL; IL-1β, 2.1 pg/mL; IL-4, 0.6 pg/mL;; IL-6, 1.5 pg/mL; IL-10, 1.1 pg/mL; IL-12 p70, 0.8 pg/mL,; TNFα, 1.1 pg/mL.

2.6 Quantitation of AFB_1-Lysine adducts in serum samples

The serum samples were analyzed using the isotope dilution mass spectrometry method previously described by Groopman and colleagues (Groopman *et al.*, 2014; McCoy *et al.*, 2005). Briefly, about 200 µL of the sample was mixed with an internal standard (10 µL × 0.1 ng AFB1-D4-lysine/mL) and pronase solution (250 µL, 13 mg/mL PBS), then incubated for 18 hours at 37 °C. The prepared serum sample-standard mixture was analyzed by ultra-performance liquid chromatography (UPLC)-tandem mass spectrometry. In this technique, the internal standard parent molecular ion [(M + H) +, m/z 461.3] is fragmented to yield an ion at m/z 398.2. The AFB_1-lysine molecular ion (m/z 457.2) is fragmented to yield an ion at m/z 394.1. A 10-point isotopic dilution standard curve was generated by triplicate injection of AFB_1-D4-lysine standard (100 pg) mixed with varying amounts of AFB_1-lysine prepared via 2-fold serial dilutions. The

data was fitted using the least squares method with a 1/x weighting factor (x= varying amounts of 0-0.4 ng AFB1-lysine). The limit of quantitation for this analysis is 0.5 pg AFB_1-lysine per milligram of albumin (McCoy *et al.*, 2005).

2.7 Data Analysis

We conducted univariate analysis on concentration data of each cytokines to test for normality of their distribution. We also calculated the descriptive statistics for the data including means, median, standard deviations and ranges. Our results revealed that the data were not normally distributed. Therefore, we used non-parametric statistical methods to conduct further analyses (Marusteri and Bacarea, 2010). The participants' cytokine concentration data were categorized by region, gender, residential status of household (urban, peri-urban, and rural) and age group (12-23, 24-35, 36-47, and 48-59 months). To compare cytokine production by region, residence type, and age group, the Kruskal-Wallis (KW) test was used. Where significant differences (stochastic dominance) among groups were found, multiple pairwise posthoc comparisons were performed with a Bonferroni type-1 error correction to analyze differences between sample pairs. In addition, the KW test was used to compare the mean ratio of IL-10 to TNFα in serum of children from different regions. The ratio of IL-10 to TNFα was calculated to assess and compare the relative risk of progression from mild to severe malaria in the different patient groups. The Mann-Whitney U-test was used to compare the mean cytokine concentrations in male and female patients. The Spearman rank correlation analysis was calculated to evaluate the monotonic relationships among the levels of cytokines in patients and in the malaria free control group. The level of significance, α was set at $p < 0.05$. The null hypothesis was rejected if the p-values were less than the adjusted α value. All analyses were performed using IBM SPSS Statistics software version 24.0 (IBM Corporation, Armonk, New York, USA).

3. Results

The results for the concentration of AFB_1-lysine adducts in serum of the Ugandan children is presented and discussed in Chapter 2 of the report. We determined that all serum samples contained detectable levels of IL-10 and IL-6. In addition, the percentage of serum samples with detectable concentrations of IFNɤ, IL-1β, and TNFα were 59%, 47%, and 46%, respectively (data not shown).

Table 13. Summary descriptive statistics for concentrations of cytokines in serum of Ugandan children with uncomplicated malaria.

Cytokine	N	Mean (pg/mL)	Median (pg/mL)
IFNɤ	158	204.7 ± 47.26	28.36 ± 59.23
IL-1β	121	40.0 ± 17.08	8.98 ± 21.41
IL-4	27	5.2 ± 1.63	2.56 ± 2.06
IL-6	267	248.4 ± 27.55	76.31 ± 34.53
IL-10	267	778.7 ± 100.15	239.11 ± 125.52
IL-12p70	24	22.4 ± 8.02	6.61 ± 10.06
TNFα	118	13.7 ± 3.30	4.87 ± 4.14

IFNɤ, Interferon gamma; IL-1β, Interleukin-1 beta; IL-4, Interleukin 4; IL-6, Interleukin 6; IL-10, Interleukin 10; IL-12p70, Interleukin 12 p70; TNFα, Tumor Necrosis Factor alpha. Cytokine concentrations recorded as Mean ± SE and Median ± SE. $P \leq 0.05$. Serum samples with concentrations below the limit of detection were excluded therefore, N the sample size for the different cytokines tested was not equal.

The majority of serum samples had undetectable levels of IL-4 and IL-12p70. We established that the concentrations of IL-4 and IL-12p70 in 99% and 91% of the serum samples respectively, were below the LOD [data not shown]. The results thus suggest that the latter group of cytokines play a limited or insignificant role in uncomplicated malaria. The measurable concentrations of

each cytokine were used to compute the descriptive statistics (Table 13). Spearman's rank correlation analysis revealed a strong positive correlation between IL-6 and IL-10 which was statistically significant, Rho = 0.77, p value < 0.0001 (Table 14). Thus, suggesting a strong positive monotonic relationship between IL-6 and IL-10 production in children with uncomplicated malaria.

Table 14. Spearman's correlation coefficients for cytokine levels in serum of children with uncomplicated malaria.

Spearman correlation coefficients for patients
Probability > |r| under H0: Rho=0
Number of Observations

	IFNɤ	IL-1β	IL-4	IL-6	IL-10	IL-12p70	TNFα
IFNɤ	1.00000	0.23252	0.29032	0.21820	0.10118	0.58752	0.12161
		0.0249	0.1688	0.0059	0.2059	0.0025	0.2705
	158	93	24	158	158	24	84
IL-1β		1.00000	0.22596	0.16445	0.01074	0.19660	0.44113
			0.2999	0.0715	0.9069	0.4825	<.0001
		121	23	121	121	15	74
IL-4			1.00000	0.28541	0.18726	0.23333	0.23824
				0.1490	0.3496	0.5457	0.3260
			27	27	27	9	19
IL-6				1.00000	0.76592	0.47673	0.22968
					<.0001	0.0185	0.0124
				267	267	24	118
IL-10					1.00000	0.29180	0.06391
						0.1665	0.4918
					267	24	118
IL-12p70						1.00000	0.00588
							0.9828
						24	16
TNFα							1.00000
							118

IFNɤ, Interferon gamma; IL-1β, Interleukin-1 beta; IL-4, Interleukin 4; IL-6, Interleukin 6; IL-10, Interleukin 10; IL-12p70, Interleukin 12 p70; TNFα, Tumor Necrosis Factor alpha. P ≤ 0.05.

3.1 Regional variations in cytokine production among patients

There were significant differences in concentration of cytokines determined in serum of children from different geographical regions (Table 15). Thus, the immune response to *P. falciparum* infection varied significantly among children living in different parts of Uganda. The mean concentration of IFNɤ in samples of children from the Western region was significantly higher than those from the Central region but not significantly different from Eastern and Northern Uganda. The mean IL-1β production by patients from Northern and Western was significantly higher than in Central Uganda. Samples of patients from Northern Uganda also had higher IL-1β concentrations than those taken from Eastern Uganda.

Table 15. Mean levels of cytokines in serum of uncomplicated malaria patients obtained from different regions of Uganda.

Cytokine	Mean Concentrations (pg/mL)				P values
	Central	Eastern	Western	Northern	
IFNɤ	166.2 ±58.86^{a}	167.0 ± 10.3.00^{ab}	347.4 ± 152.68^{bc}	193.2 ± 89.59^{ab}	0.024
IL-1β	6.9 ± 0.91^{a}	16.1 ± 6.89^{ab}	104.0 ± 78.90^{bc}	73.0 ± 47.92^{c}	0.000
IL-6	222.0 ± 58.42^{a}	202.9 ± 34.50^{a}	592.4 ± 122.29^{b}	178.0 ± 37.21^{a}	0.000
IL-10	647.2 ± 221.09^{a}	454.0 ± 64.33^{a}	1846.4 ± 436.47^{b}	785.2 ± 182.40^{a}	0.000
TNFα	5.2 ± 0.91^{a}	26.2 ± 10.93^{ab}	11.2 ± 3.26^{ab}	10.1 ± 1.79^{b}	0.038

IFNɤ, Interferon gamma; IL-1β, Interleukin-1 beta; IL-6, Interleukin 6; IL-10, Interleukin 10; IL-13, Interleukin 13; TNFα, Tumor Necrosis Factor alpha. Cytokine concentrations recorded as mean ± SEM. Groups with different letters are significantly different (Kruskal-Wallis test and Dunn's posthoc multiple pairwise comparison test, $P \leq 0.05$).

The mean IL-6 and IL-10 concentrations in samples from Western Uganda were significantly higher than the concentrations in Central, Eastern and Northern Uganda samples.For TNFα, samples from the Northern region contained a significantly higher mean concentration than those

from the Central region. Similar to the Eastern-Western-Northern group, there were no significant differences among the TNFα concentrations in samples from Central, Eastern, and Western Uganda.

There were also significant differences in the level of IL-10 produced relative to TNFα in patients from the different parts of Uganda. The ratio of IL-10: TNFα have been previously suggested to infer the relative risk of progression from uncomplicated to severe malaria among patients (May *et al.*, 2000; Othoro *et al.*, 1999). The mean ratios of IL-10: TNFα concentrations calculated were 472, 233, 172, and 100 for the Western, Central, Northern, and Eastern regions, respectively. The KW test showed that the mean ratios of IL-10 to TNFα varied significantly across regions, chi-square test statistic = 8.98, p-value = 0.0295 (Fig 9). Subsequently, the Tukey's Honest Significant Difference posthoc analysis revealed that the mean IL-10: TNFα ratios for the Western region were significantly higher than those of the Eastern region but not the Central and Northern regions.

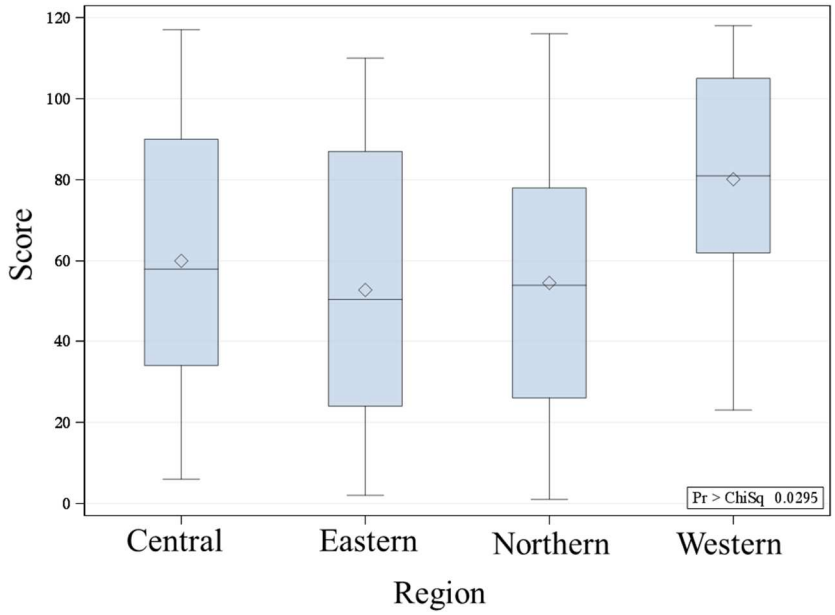


Figure 9. The distribution of Wilcoxon scores for the ratio of the concentration of IL-10 to TNFα in serum of children aged 12-59 months with uncomplicated malaria, sampled from four regions of Uganda

3.2 Effect of residence type on immune response of children with uncomplicated malaria

With the exception of IL-1β whose concentration was significantly higher in serum samples from rural areas than in urban and peri-urban areas (KW test statistic = 11.7, p-value = 0.003), the IFNɤ, IL-4, IL-6, IL-10, IL-12p70, and TNFα concentrations were similar among patients from urban, peri-urban, and rural settings (data not shown). These results indicate that, the type of residence does not play a significant role in determining the magnitude of immune response in children with uncomplicated malaria.

3.3 Effect of age group and gender differences on mean cytokine production

Inter age-group comparisons using the KW multiple comparisons test revealed that there were no significant differences in cytokine production among the different uncomplicated malaria patients' age groups. Similarly, the Mann-Whitney U-test conducted to compare cytokine

production in male and female patients did not reveal any significant differences; the mean concentrations of IFNɤ, IL-1β, IL-4, IL-6, IL-10, IL-12p70, and TNFα cytokines produced by male participants with uncomplicated malaria were similar to those produced by their female counterparts (data not shown). Thus, age-group and gender differences among children between 1 and 5 years did not influence the magnitude of immune response in uncomplicated malaria patients.

3.4 The relationship between aflatoxin exposure and immune response

The scatter plots in Fig. 10 illustrate the distribution of cytokine concentrations corresponding to AFB_1 adduct levels of each malaria patient. The results of the Pearson correlation analysis are summarized in Table 16.

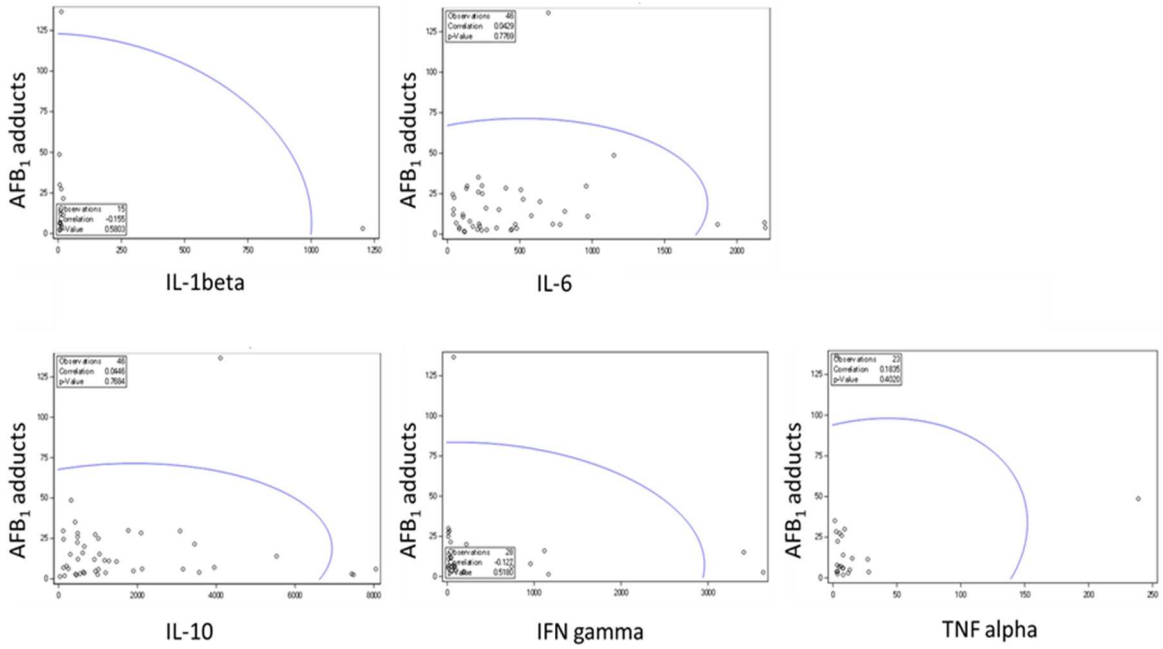


Figure 10. The concentrations of cytokines and AFB1 adducts in serum of Ugandan children aged 1-5 years with uncomplicated malaria infection

Table 16. The association between the level of AFB1-lysine adducts and concentration of cytokines in serum of Ugandan children with uncomplicated malaria.

Cytokine	Number of Participants	Pearson's Correlation Coefficient, Rho	P value
IFN gamma	28	-0.1275	0.5180
IL-1 beta	15	-0.1554	0.5803
IL-6	46	0.0429	0.7769
IL-10	46	0.0446	0.7684
TNF alpha	23	0.1835	0.4020

IFN, Interferon; IL, Interleukin; TNF, Tumor Necrosis Factor. $P \leq 0.05$. Serum samples with concentrations below the limit of detection were excluded therefore, sample sizes for the different cytokines tested were not equal.

The Pearson's correlation coefficients calculated indicate that there is no correlation between the concentrations of AFB1 adducts and the concentrations of IFNγ, IL1β, IL6, IL10, and TNFα. Therefore, the results of our study suggest that the immune response in Ugandan children with uncomplicated malaria is not associated with the level of exposure to aflatoxins.

4. Discussion

In this study, we determined the concentrations of a range of cytokines in the serum of Ugandan children aged between 1 and 5 years with uncomplicated malaria. The goal was to characterize the immunological milieu and how it relates to pathological outcomes of *P. falciparum* infection. Numerous past studies have demonstrated that although inflammation is essential during an immune response, uncontrolled inflammation can be deleterious to health. Well controlled inflammation is necessary to control *P. falciparum* multiplication and to destroy invading parasites (Torre *et al.*, 2002). However, dysregulated cytokine production may cause either ineffective parasite clearance or excessive inflammation which results in tissue damage and severe disease (Angulo and Fresno, 2002; Gun *et al.*, 2014; Mackroth *et al.*, 2016; Perez-Mazliah and Langhorne, 2015). Therefore, a careful balance between pro- and anti-inflammatory

cytokines is vital for an optimal host immune response. Here, we discuss the levels of the different cytokines determined in serum of Ugandan patients with uncomplicated malaria.

Malaria patients typically present with elevated levels of IL-10 in their blood that tends to inhibit the production of proinflammatory cytokines such as TNFα, IL-1β, and IL-6 (Boeuf *et al.*, 2012; Ho *et al.*, 1998; Peyron *et al.*, 1994). Our study showed that the levels of IL-1β, IL-6, and IL10 were significantly expressed while IFNɤ, IL-4, IL-12p70, and TNFα serum levels were low and mostly undetectable in serum of patients. Previous studies have shown that a substantial amount of IL-10 is produced by a variety of T cells including Th1, Th2, regulatory T cells and cells of the myeloid lineage (Jagannathan *et al.*, 2014; Niikura *et al.*, 2011; Peyron *et al.*, 1994). Similar results were obtained in earlier studies on Togolese and Kenyan children which reported strongly elevated levels of IL-10 in malaria patients as compared to asymptomatic controls (Ayimba *et al.*, 2011). Interestingly, the concentrations of IL-10 measured in our study were comparable to those among uncomplicated malaria children in previous studies (Nyirenda *et al.*, 2015; Peyron *et al.*, 1994). Moreover, IL-10 acts on monocytes and macrophages to suppress the production of IFNɤ and IL-12 albeit a delicate balance is critical since IFNɤ suppression favors continued parasite multiplication and maturation (Niikura *et al.*, 2011; Torre *et al.*, 2002). Our study showed that patients with uncomplicated malaria are still capable of producing large amounts of IFNɤ in their blood. This could be explained by studies conducted on malaria patients taken from high transmission areas which have shown that IFNɤ/IL-10 co-producing $CD4^+$ T cells dominate immune responses and aid to limit pathology in patients with uncomplicated malaria. However, the $CD4^+$ T cells do not protect against subsequent *P. falciparum* infection (Jagannathan *et al.*, 2014). These findings are further supported by a recent study that demonstrated that malaria infection upregulates expression of antigen-specific CTLA4 and PD1 receptors by $CD4^+$ effector

T cells that simultaneously produce IFNɤ and IL-10 (Mackroth *et al.*, 2016). These $CTLA4^{+}PD1^{+}CD4^{+}$ T cells acts as an off switch that controls inflammatory activity of the expressing cells thus protecting individuals against excessive inflammation (Mackroth *et al.*, 2016; McCoy and Le Gros, 1999). High IL-10 also inhibits the synthesis of IL-4 by Th2 cells (Niikura *et al.*, 2011).

Our study found that about 90% of serum samples had undetectable concentrations of IL-12p70. Moreover, the samples with detectable IL-12p70 were marginally higher than the LOD suggesting that IL-12p70 does not play a significant role in uncomplicated malaria pathology. These results are corroborated by a microarray-based network analysis study by Hu and coworkers (Hu, 2013), that investigated genetic immunological circuitry and illustrated that gene networks centered on IL-6, IL-17 and transforming growth factor beta (TGFβ) play a major regulatory role in malaria pathology but not IL-12. The same study also showed that the moderate to mild level of IFNɤ observed in malaria patients is achieved via a pathway different from the classical IL-12 Th1 pattern (Hu, 2013). However, this may not hold true for all age groups as some studies in adult patients with uncomplicated malaria showed that IL-12 levels increase during acute response to infection (Torre *et al.*, 2002).

The concentrations of IL-4 in serum of the majority of patients were below the LOD despite previous reports of Th2 immune responses in malaria patients (Angulo and Fresno, 2002; Torre *et al.*, 2002). There is evidence for IL-4/IFNɤ co-production by biphasic $CD4^{+}$ T cells during the erythrocytic stage of *P. chabaudi* infection in mice (Perez-Mazliah and Langhorne, 2015). The resulting IL-4 production drives CD4+ T cell polarization to Th2 phenotypes via a GATA3 transcription factor which leads to production of Th2 cytokines including IL-4, IL-5, and IL-13 (Perez-Mazliah and Langhorne, 2015). Interleukin-4 activates STAT6 via a positive feedback

mechanism that results in further production of IL-4 (Hu, 2013; Silva-Filho *et al.*, 2014). Thus, we would anticipate that higher frequencies and concentrations of IL-4 would be found in serum of patients with uncomplicated malaria. However, the vastly undetectable levels of IL-4 agree, and are supported by Hu's microarray-based study that showed that the IL-4 producing Th2 type immune response tends to be suboptimal as compared to Th1 responses and that the levels of Th2 cytokines remained unchanged by *P. falciparum* infection. Hence, they play no significant role in uncomplicated malaria pathology (Hu, 2013).

The results of our study indicated that the mean levels of cytokines did not significantly vary by gender or age of participants. This suggests that these factors do not significantly influence malaria outcomes in children between 1 and 5 years of age. Our findings are consistent with a previous study among children in Tororo, a hyper-endemic district of Uganda, which reported that; 1) the relative risk of malaria transmission among children between 1-3 years was the same, and 2) age-dependent differences in parasitaemia were not significant (Jagannathan *et al.*, 2012). This resemblance could be partly attributed to the lack of a fully developed clinical immunity against *P. falciparum* before the age of five (Crompton *et al.*, 2014). In areas of intense malaria transmission, children eventually acquire natural immunity against severe malaria but remain susceptible to infection and subsequently develop mild malaria (Doolan *et al.*, 2009). Nonetheless, the protection is a cumulative outcome of multiple infections over a long time resulting in a sufficiently diverse repertoire of *P. falciparum* strain specific responses (Doolan *et al.*, 2009). Similar to gender and age, the residence type of the household did not significantly influence pathological outcomes of malaria in children. Immune responses among children living in urban, peri-urban, and rural settings were similar except for IL-1β in rural children which were significantly higher than both urban and peri-urban children. The reason for the difference in IL-

1β between rural and urban patients is unclear although it could be due to failure to seek treatment in a timely manner. In Uganda, the overall prevalence of malaria is higher in rural areas than in urban settings (21% versus 6%, respectively) but the percentage of children for whom treatment is sought is lower in rural than urban settings, i.e. 81% in rural as compared to 90% in urban settings (Rwashana *et al.*, 2015).

The prevalence of malaria among children under the age of five in the regions of Uganda studies here are 36%: Eastern, 19.6%: Northern, 14%: Central and 4%: Western (Rwashana *et al.*, 2015). We thus hypothesized that the immunological milieu in serum of malaria patients living in high transmission areas such as the Eastern and Northern Uganda would be more anti-inflammatory than in children from lower transmission areas of Central and Western Uganda. Our results instead showed that children living in low malaria transmission areas of Western Uganda had the highest levels of IL-10 as well as the highest IL-10: TNFα ratio. A low IL-10: TNFα cytokine ratio has been associated with excessive inflammation and subsequently more severe malaria (Mbengue *et al.*, 2015; Othoro *et al.*, 1999; Perera *et al.*, 2013). Therefore, an adequate IL-10 response appears to be necessary to regulate proinflammatory cytokine activity in malaria patients (Perera *et al.*, 2013). In the regions studied, we ranked the ratios of IL-10: TNFα from highest to lowest: the Western region was highest followed in order by Central, Northern and Eastern regions, respectively. It is thus conceivable that the risk of progression from mild to severe malaria would follow a similar order; the risk is highest among children living in Eastern and lowest among those living in Western Uganda. These differences could be attributed to a variety of region specific factors such as environmental contaminants that alter immune function (MacGillivray and Kollmann, 2014), dietary imbalances and nutritional deficiencies especially micronutrient limitations that affect immune competence (Ibrahim and El-Sayed, 2015;

MacGillivray and Kollmann, 2014; Prasad, 2013), and exposure to foodborne mycotoxins (Bryden, 2007; Wu *et al.*, 2014b). Therefore, identifying area specific environmental factors that modulate immune system function is important for identifying interventions that promote long-term protection from disease.

5. Conclusions

Our study reveals key cytokine production patterns among uncomplicated malaria patients that may be useful to predict clinical outcomes of infection in children. These include a strong expression of IL-1β, IL-6 and IL-10 in serum, coupled with suppressed production of IFNɣ, IL-4, IL-12p70, and TNFα. In addition, the mean cytokine production varies from one geographical region to another, and is related to malaria endemicity. The household residential status, age group, and gender differences do not affect the immune response to *P. falciparum* infection among children between 1 and 5 years of age. Therefore, identifying region-specific factors that modulate immune system function is important to promote long-term protection against severe malaria in children. Finally, we recommend that future studies on immune response among children with uncomplicated malaria in Uganda and endemically similar communities to include other pro- and anti-inflammatory cytokines such as IL-17A, IL-13, and TGFβ in order to generate a more complete cytokine fingerprint for patients between 1 and 5 years of age. Nonetheless, the results of this study provide vital information regarding the cytokine profile in Ugandan children with uncomplicated malaria. We hope that in the future, these could be used by clinicians to determine which children are at a higher risk than others of progressing from mild to severe malaria.

APPENDIX B: Study questionnaire

The association between serum levels of AFB_1 biomarkers and cytokine concentration among Ugandan children aged 1-5 years with uncomplicated malaria

Child Name: ……..

Child or Patient Identification code: ……..

Name of hospital ……..… Date ……..

Name of interviewer ……

A. Child's caretaker information (*only parents & adult legal guardians can give consent***)**

1. What is the relationship between you (caretaker) and the child? ……
2. Please indicate your age in years ……*Years*
3. How long have you lived with this child presenting with malaria? ……..…...… *months*
4. How far do you stay in relation to the hospital facility? ……..…….……*Km*
5. How would you best describe the residence of the patient?

   Rural [ ] Peri-urban [ ] Urban [ ]

B. General characteristics of patient

1. Age in Months …… *Months*
2. Weight …… *Kilograms*
3. Gender *(tick)* Male [ ] Female [ ]

C. Record of clinical features (history and presenting symptoms) tick all that apply

Clinical feature	Yes	No	Clinical feature	Yes	No
Fever	[ ]	[ ]	Cerebral malaria	[ ]	[ ]
Cough	[ ]	[ ]	Severe anemia	[ ]	[ ]
Vomiting	[ ]	[ ]	Renal failure	[ ]	[ ]
Headache	[ ]	[ ]	Pulmonary edema	[ ]	[ ]
Muscle pain	[ ]	[ ]	Hypoglycemia	[ ]	[ ]
Abdominal discomfort	[ ]	[ ]	Shock	[ ]	[ ]
			Spontaneous		
Fatigue	[ ]	[ ]	bleeding	[ ]	[ ]
			Repeated		
Chills	[ ]	[ ]	convulsions	[ ]	[ ]
Nausea	[ ]	[ ]	Acidosis	[ ]	[ ]
Pallor	[ ]	[ ]	Hemoglobinuria	[ ]	[ ]

D. Record of treatment prior to hospital visit

1. What was the date of symptoms onset? *dd/mm/yyyy* ……/……/……
2. When was malaria diagnosed? *dd/mm/yyyy* ……/……/……
3. Was the child given any medication before coming to hospital? Yes [ ] No [ ]
4. Was your child referred to this facility from another health center or clinic? Yes [ ] No [ ]
5. If yes in (3) above, was the child given any pre-referral medication? Yes [ ] No [ ]
6. What type of medication was he/she given? (*Tick all that applies)*

Injectable anti-malarial treatment *(on buttocks, thighs*) [ ]

Intravenous injection (*in a drip*) [ ]

Oral anti-malarial medication [ ]

Fever/ pain relief anti-inflammatory medicines [ ]

Others, Specify

…….

E. Malaria History of Patient

1. Prior to this visit, has your child ever had confirmed malaria before?

   Yes [ ] No [ ] Don't know [ ]

2. If yes, when? *Give month/year* ……

3. How was it treated? *Tick all that applies*

   Injectable anti-malarial treatment (on buttocks, thighs) [ ]

   Intravenous injection (in a drip) [ ]

   Oral anti-malarial medication [ ]

   Others, Specify

   ……..

4. Was the anti-malarial treatment dose complete? Yes [ ] No [ ]

5. Prior to this visit, has your child ever had symptoms consistent with malaria but never formally diagnosed? Yes [ ] No [ ] don't know [ ]

6. If yes, when? *Give month/year* ……

7. How were the symptoms treated? *Tick all that applies*

   Injectable anti-malarial (*on buttocks, thighs*) [ ]

   Intravenous injection (*in a drip*) [ ]

Oral anti-malarial medication [ ]

Fever/ pain relief anti-inflammatory medicines [ ]

Others, Specify

……..

F. Immune disorders and Co-infections

1. Does the child have any systemic or localized autoimmune disease?

   Yes [ ] No [ ] don't know [ ]

2. Is the child suffering from any other infections besides malaria? Yes [ ] No [ ] Don't know [ ]
3. Has the child been tested for HIV before? Yes [ ] No [ ] Don't know [ ]
4. If yes, what were the results? Negative [ ] Positive [ ]

G. Laboratory investigation and results

1. Malaria parasite test ordered: Yes [ ] No [ ]
2. Malaria Rapid Diagnostic Tests (RDT) done: Yes [ ] No [ ]
3. Malaria microscopy done: Yes [ ] No [ ]

4. If yes, which of the following techniques was used?

   Blood smear thin film [ ] Blood smear thick film [ ]

   Please indicate the result in (3) above: ……. (Parasite load or number)

5. Severe Anemia: Hematocrit <21% or Hb <7 g/dL: Yes [ ] No [ ]

6. If Yes, indicate figure (% or g/dL): …….

H. OFFICIAL USE ONLY (Do not fill).

1. Immunological tests done Yes [ ] No [ ]
2. Aflatoxin B1 biomarkers determined Yes [ ] No [ ]
3. If Yes in H-2 above, indicate concentration: …… pg AFB_1- lysine /mg albumin

BIBLIOGRAPHY

BIBLIOGRAPHY

Abbas, H., Yoshizawa, T. and Shier, W., 2013. Cytotoxicity and phytotoxicity of trichothecene mycotoxins produced by *Fusarium* Toxicon 74: 68-75.

Abdulrazzaq, Y.M., Osman, N. and Ibrahim, A., 2002. Fetal exposure to aflatoxins in the United Arab Emirates. Annals of tropical paediatrics 22: 3-9.

Ahangarkani, F., Rouhi, S. and Gholamour Azizi, I., 2014. A review on incidence and toxicity of fumonisins. Toxin Reviews 33: 95-100.

Alassane-Kpembi, I., Puel, O. and Oswald, I., 2014. Toxicological interactions between the mycotoxins deoxynivalenol, nivalenol and their acetylated derivatives in intestinal epithelial cells. Archives of toxicology 89: 1337-1346.

Alexander, N.J., McCormick, S.P. and Ziegenhorn, S.L., 1999. Phytotoxicity of selected trichothecenes using Chlamydomonas reinhardtii as a model system. Natural Toxins 7: 265-269.

Angulo, I. and Fresno, M., 2002. Cytokines in the pathogenesis of and protection against malaria. Clinical and diagnostic laboratory immunology 9: 1145-1152.

Argos, M., Ahsan, H. and Graziano, J.H., 2012. Arsenic and human health: epidemiologic progress and public health implications. Rev Environ Health 27: 191-195.

Arimond, M. and Ruel, M.T., 2004. Dietary diversity is associated with child nutritional status: evidence from 11 demographic and health surveys. J Nutr 134: 2579-2585.

Asiki, G., Seeley, J., Srey, C., Baisley, K., Lightfoot, T., Archileo, K., Agol, D., Abaasa, A., Wakeham, K. and Routledge, M.N., 2014. A pilot study to evaluate aflatoxin exposure in a rural Ugandan population. Tropical Medicine & International Health 19: 592-599.

Avantaggiato, G., Havenaar, R. and Visconti, A., 2004. Evaluation of the intestinal absorption of deoxynivalenol and nivalenol by an in vitro gastrointestinal model, and the binding efficacy of activated carbon and other adsorbent materials. Food and Chemical Toxicology 42: 817-824.

Ayimba, E., Hegewald, J., Ségbéna, A., Gantin, R., Lechner, C., Agosssou, A., Banla, M. and Soboslay, P., 2011. Proinflammatory and regulatory cytokines and chemokines in infants with uncomplicated and severe Plasmodium falciparum malaria. Clinical & Experimental Immunology 166: 218-226.

Azziz-Baumgartner, E., Lindblade, K., Gieseker, K., Rogers, H.S., Kieszak, S., Njapau, H., Schleicher, R., McCoy, L.F., Misore, A. and DeCock, K., 2005. Case-control study of an acute aflatoxicosis outbreak, Kenya, 2004. Environ Health Perspect: 1779-1783.

Banea, J.P., Bradbury, J.H., Mandombi, C., Nahimana, D., Denton, I.C., Kuwa, N. and Tshala Katumbay, D., 2013. Control of konzo by detoxification of cassava flour in three villages in the Democratic Republic of Congo. Food Chem Toxicol 60: 506-513.

Barchowsky, A., Cartwright, I.L., Reichard, J.F., Futscher, B.W. and Lantz, R.C., 2011. Arsenic toxicology: translating between experimental models and human pathology. Environ Health Perspect 119: 1356-1363.

Bartoloni, A. and Zammarchi, L., 2012. Clinical aspects of uncomplicated and severe malaria. Mediterranean journal of hematology and infectious diseases 4: 1-10.

Basu, A., Mitra, S., Chung, J., Guha Mazumder, D.N., Ghosh, N., Kalman, D., von Ehrenstein, O.S., Steinmaus, C., Liaw, J. and Smith, A.H., 2011. Creatinine, diet, micronutrients, and arsenic methylation in West Bengal, India. Environ Health Perspect 119: 1308-1313.

Bbosa, G.S., Lubega, A., Kyegombe, D.B., Kitya, D., Ogwal-Okeng, J. and Anokbonggo, W.W., 2013. Review of the biological and health effects of aflatoxins on body organs and body systems. INTECH Open Access Publisher.

Becker, D.E., 2010. Nausea, vomiting, and hiccups: a review of mechanisms and treatment. Anesthesia progress 57: 150-157.

Bedard, L.L. and Massey, T.E., 2006. Aflatoxin B1-induced DNA damage and its repair. Cancer Lett 241: 174-183.

Bejon, P., Andrews, L., Hunt-Cooke, A., Sanderson, F., Gilbert, S.C. and Hill, A., 2006. Thick blood film examination for Plasmodium falciparum malaria has reduced sensitivity and underestimates parasite density. Malar J 5: 104-107.

Bellio, A., Bianchi, D.M., Gramaglia, M., Loria, A., Nucera, D., Gallina, S., Gili, M. and Decastelli, L., 2016. Aflatoxin M1 in Cow's Milk: Method Validation for Milk Sampled in Northern Italy. Toxins 8: 57-68.

Bennett, J. and Inamdar, A., 2015. Are Some Fungal Volatile Organic Compounds (VOCs) Mycotoxins? Toxins 7: 3785-3804.

Bennett, J.W. and Klich, M., 2003. Mycotoxins. Clin Microbiol Rev 16: 497-516.

Berek, L., Petri, I., Mesterhazy, A., Téren, J. and Molnár, J., 2001. Effects of mycotoxins on human immune functions in vitro. Toxicology in vitro 15: 25-30.

Bertolino, P. and Bowen, D.G., 2015. Malaria and the liver: immunological hide-and-seek or subversion of immunity from within? Frontiers in microbiology 6: 41-55.

Bertuzzi, T., Camardo Leggieri, M., Battilani, P. and Pietri, A., 2014a. Co-occurrence of type A and B trichothecenes and zearalenone in wheat grown in northern Italy over the years 2009-2011. Food Addit Contam Part B Surveill 7: 273-281.

Bertuzzi, T., Leggieri, M.C., Battilani, P. and Pietri, A., 2014b. Co-occurrence of type A and B trichothecenes and zearalenone in wheat grown in northern Italy over the years 2009–2011. Food Additives & Contaminants: Part B 7: 273-281.

Bhat, R., Ramakrishna, Y., Beedu, S. and Munshi, K., 1989. Outbreak of trichothecene mycotoxicosis associated with consumption of mould-damaged wheat products in Kashmir Valley, India. The Lancet 333: 35-37.

Bianco, G., Fontanella, B., Severino, L., Quaroni, A., Autore, G. and Marzocco, S., 2012. Nivalenol and deoxynivalenol affect rat intestinal epithelial cells: a concentration related study. 7: e52051.

Boeuf, P.S., Loizon, S., Awandare, G.A., Tetteh, J., Addae, M.M., Adjei, G.O., Goka, B., Kurtzhals, J., Puijalon, O. and Hviid, L., 2012. Insights into deregulated TNF and IL-10 production in malaria: implications for understanding severe malarial anaemia. Malar J 11: 253-260.

Boivin, M.J., Okitundu, D., Makila-Mabe Bumoko, G., Sombo, M.T., Mumba, D., Tylleskar, T., Page, C.F., Tamfum Muyembe, J.J. and Tshala-Katumbay, D., 2013. Neuropsychological effects of konzo: a neuromotor disease associated with poorly processed cassava. Pediatrics 131: 1231-1239.

Bowers, E.L. and Munkvold, G.P., 2014. Fumonisins in Conventional and Transgenic, Insect-Resistant Maize Intended for Fuel Ethanol Production: Implications for Fermentation Efficiency and DDGS Co-Product Quality. Toxins 6: 2804-2825.

Bradburn, N., Coker, R.D. and Blunden, G., 1994. The aetiology of turkey 'X'disease. Phytochemistry 35: 817.

Brand-Miller, J.C., Stockmann, K., Atkinson, F., Petocz, P. and Denyer, G., 2009. Glycemic index, postprandial glycemia, and the shape of the curve in healthy subjects: analysis of a database of more than 1000 foods. Am J Clin Nutr 89: 97-105.

Bren, U., Fuchs, J.E. and Oostenbrink, C., 2014. Cooperative binding of aflatoxin B1 by cytochrome P450 3A4: A computational study. Chemical research in toxicology 27: 2136-2147.

Broekaert, N., Devreese, M., De Mil, T., Fraeyman, S., Antonissen, G., De Baere, S., De Backer, P., Vermeulen, A. and Croubels, S., 2015. Oral Bioavailability, Hydrolysis, and Comparative Toxicokinetics of 3-Acetyldeoxynivalenol and 15-Acetyldeoxynivalenol in Broiler Chickens and Pigs. Journal of agricultural and food chemistry 63: 8734-8742.

Bryden, W.L., 2007. Mycotoxins in the food chain: human health implications. Asia Pacific journal of clinical nutrition 16: 95-101.

Bullerman, L.B. and Bianchini, A., 2007. Stability of mycotoxins during food processing. International Journal of food microbiology 119: 140-146.

Burel, C., Tanguy, M., Guerre, P., Boilletot, E., Cariolet, R., Queguiner, M., Postollec, G., Pinton, P., Salvat, G. and Oswald, I.P., 2013. Effect of low dose of fumonisins on pig health: Immune status, intestinal microbiota and sensitivity to Salmonella. Toxins 5: 841-864.

Canady, R.A., Coker, R.D., Egan, S.K., Krska, R., Kuiper-Goodman, T., Pestka, J.J., Olsen, M., Resnik, S. and Schlatter, J., 2001a. Safety evaluation of certain mycotoxins in food. Deoxynivalenol. Fifth report of the Joint FAO/WHO Expert Committee on Food Additives. , 74. Food & Agriculture Org.

Canady, R.A., Coker, R.D., Egan, S.K., Krska, R., Olsen, M., Resnik, S. and Schlatter, J., 2001b. T-2 and HT-2 toxins. Safety Evaluation of Certain Mycotoxins in Food. Joint FAO/WHO Expert Committee on Food Additives (JECFA) 47: 557-652.

Cappozzo, J., Jackson, L., Lee, H.J., Zhou, W., Al-Taher, F., Zweigenbaum, J. and Ryu, D., 2017. Occurrence of Ochratoxin A in Infant Foods in the United States. Journal of Food Protection 80: 251-256.

Cardwell, K., Desjardins, A., Henry, S., Munkvold, G. and Robens, J., 2001. Mycotoxins: the cost of achieving food security and food quality. American Phytopathological Society.

Carstensen, M., Thomsen, C. and Hermansen, K., 2003. Incremental area under response curve more accurately describes the triglyceride response to an oral fat load in both healthy and type 2 diabetic subjects. Metabolism 52: 1034-1037.

Castegnaro, M., Canadas, D., Vrabcheva, T., Petkova-Bocharova, T., Chernozemsky, I.N. and Pfohl-Leszkowicz, A., 2006. Balkan endemic nephropathy: role of ochratoxins A through biomarkers. Molecular nutrition & food research 50: 519-529.

Cavaillon, J.-M., 2001. Pro-versus anti-inflammatory cytokines: myth or reality. CELLULAR AND MOLECULAR BIOLOGY-PARIS-WEGMANN- 47: 695-702.

Chen, J.G., Egner, P.A., Ng, D., Jacobson, L.P., Munoz, A., Zhu, Y.R., Qian, G.S., Wu, F., Yuan, J.M., Groopman, J.D. and Kensler, T.W., 2013. Reduced aflatoxin exposure presages decline in liver cancer mortality in an endemic region of China. Cancer Prev Res (Phila) 6: 1038-1045.

Commission, E., 2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuff. 2006R1881-EN-01.09. 2014-014.001-1.

Conrady-Lorck, S., Gareis, M., Feng, X.-C., Amselgruber, W., Forth, W. and Fichtl, B., 1988. Metabolism of T-2 toxin in vascularly autoperfused jejunal loops of rats. Toxicology and applied pharmacology 94: 23-33.

Cotty, P.J. and Jaime-Garcia, R., 2007. Influences of climate on aflatoxin producing fungi and aflatoxin contamination. International Journal of food microbiology 119: 109-115.

Crompton, P.D., Moebius, J., Portugal, S., Waisberg, M., Hart, G., Garver, L.S., Miller, L.H., Barillas, C. and Pierce, S.K., 2014. Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. Annual review of immunology 32: 157-187.

Crump, K.S., 1995. Calculation of benchmark doses from continuous data. Risk Analysis 15: 79-89.

Cundliffe, E. and Davies, J.E., 1977. Inhibition of initiation, elongation, and termination of eukaryotic protein synthesis by trichothecene fungal toxins. Antimicrobial agents and chemotherapy 11: 491-499.

Dashwood, R., Negishi, T., Hayatsu, H., Breinholt, V., Hendricks, J. and Bailey, G., 1998. Chemopreventive properties of chlorophylls towards aflatoxin B1: a review of the antimutagenicity and anticarcinogenicity data in rainbow trout. Mutat Res 399: 245-253.

Davis, A.S., Hill, J.D., Chase, C.A., Johanns, A.M. and Liebman, M., 2012. Increasing cropping system diversity balances productivity, profitability and environmental health. PLoS One 7: e47149.

Desjardins, A.E., McCormick, S.P. and Appell, M., 2007. Structure-activity relationships of trichothecene toxins in an Arabidopsis thaliana leaf assay. Journal of agricultural and food chemistry 55: 6487-6492.

Dhanasekaran, D., Shanmugapriya, S., Thajuddin, N. and Panneerselvam, A., 2011. Aflatoxins and aflatoxicosis in human and animals, Aflatoxins-Biochemistry and Molecular Biology. InTech.

Diao, E., Dong, H., Hou, H., Zhang, Z., Ji, N. and Ma, W., 2015. Factors influencing aflatoxin contamination in before and after harvest peanuts: a review. Journal of Food Research 4: 148-154.

Diaz, G. and Sánchez, M., 2015. Determination of aflatoxin M1 in breast milk as a biomarker of maternal and infant exposure in Colombia. Food additives & contaminants: part A 32: 1192-1198.

Dietrich, B., Ramchandran, K. and Von Roenn, J.H., 2015. Nausea and Vomiting. Psycho-Oncology: 199.

Dima, E., Koltsida, O., Katsaounou, P., Vakali, S., Koutsoukou, A., Koulouris, N.G. and Rovina, N., 2015. Implication of Interleukin (IL)-18 in the pathogenesis of chronic obstructive pulmonary disease (COPD). Cytokine 74: 313-317.

Dinarello, C.A., 2000. Proinflammatory cytokines. Chest Journal 118: 503-508.

Dinolfo, M.I. and Stenglein, S.A., 2014. Fusarium poae and mycotoxins: potential risk for consumers. Boletín de la Sociedad Argentina de Botánica 49: 5-20.

Dohnal, V., Wu, Q. and Kuča, K., 2014. Metabolism of aflatoxins: key enzymes and interindividual as well as interspecies differences. Archives of toxicology 88: 1635-1644.

Doolan, D.L., Dobaño, C. and Baird, J.K., 2009. Acquired immunity to malaria. Clinical microbiology reviews 22: 13-36.

Eaton, D.L. and Gallagher, E.P., 1994. Mechanisms of aflatoxin carcinogenesis. Annu Rev Pharmacol Toxicol 34: 135-172.

EFSA, 2009. Scientific opinion on arsenic in food - European Food Safety Authority (EFSA) CONTAM Panel. EFSA Journal 7: 1351-1529.

EFSA, 2011. Scientific Opinion on the risks for public health related to the presence of zearalenone in food. Conclusions and recommendations. EFSA J 9: 86-88.

EFSA, 2013a. Deoxynivalenol in food and feed: occurrence and exposure. EFSA J. 11: 3379-3435.

EFSA, 2013b. Scientific Opinion on risks for animal and public health related to the presence of nivalenol in food and feed. EFSA Journal 11: 3262-3380.

Egner, P.A., Wang, J.B., Zhu, Y.R., Zhang, B.C., Wu, Y., Zhang, Q.N., Qian, G.S., Kuang, S.Y., Gange, S.J., Jacobson, L.P., Helzlsouer, K.J., Bailey, G.S., Groopman, J.D. and Kensler, T.W., 2001. Chlorophyllin intervention reduces aflatoxin-DNA adducts in individuals at high risk for liver cancer. Proc Natl Acad Sci U S A 98: 14601-14606.

Eriksen, G.S., Pettersson, H. and Lundh, T., 2004. Comparative cytotoxicity of deoxynivalenol, nivalenol, their acetylated derivatives and de-epoxy metabolites. Food and Chemical Toxicology 42: 619-624.

Escriva, L., Font, G. and Manyes, L., 2015. In vivo toxicity studies of *Fusarium* mycotoxins in the last decade: a review. Food Chem Toxicol 15: 44-47.

Escrivá, L., Font, G. and Manyes, L., 2015. In vivo toxicity studies of fusarium mycotoxins in the last decade: A review. Food and Chemical Toxicology 78: 185-206.

Ey Chua, E.Y., Zalilah, M.S., Ys Chin, Y.S. and Norhasmah, S., 2012. Dietary diversity is associated with nutritional status of Orang Asli children in Krau Wildlife Reserve, Pahang. Malays J Nutr 18: 1-13.

Fallah, A.A., Barani, A. and Nasiri, Z., 2015. Aflatoxin M1 in raw milk in Qazvin province, Iran: a seasonal study. Food Additives & Contaminants: Part B 8: 195-198.

FAO., 2004. Worldwide regulations for mycotoxins in food and feed in 2003. FAO.

FDA, 2001. Guidance for industry: fumonisin levels in human foods and animal feeds; final guidance. In: U.F. US Food and Drug Administration (Ed.).

FDA, 2010. Guidance for industry and FDA: Advisory levels for deoxynivalenol (DON) in finished wheat products for human consumption and grains and grain by-products used for animal feed, US Food and Drug Administration: Silver Spring, MD, USA.

Feeley, M., Agudo, A., Bronson, R., Edgar, J., Grant, D., Hambridge, T. and Schlatter, J., 2012. Cyanogenic glycosides. Joint FAO/WHO Expert Committee on Food Additives (JECFA): Safety Evaluation of Certain Food Additives and Contaminants 65: 171-323.

Foroud, N.A. and Eudes, F., 2009. Trichothecenes in cereal grains. International journal of molecular sciences 10: 147-173.

Frison, E.A., Smith, I.F., Johns, T., Cherfas, J. and Eyzaguirre, P.B., 2006. Agricultural biodiversity, nutrition, and health: making a difference to hunger and nutrition in the developing world. Food and nutrition bulletin 27: 167-179.

Frosch, A.E. and John, C.C., 2012. Immunomodulation in Plasmodium falciparum malaria: experiments in nature and their conflicting implications for potential therapeutic agents. Expert review of anti-infective therapy 10: 1343-1356.

Funk, C.C., Rowland, J., Eilerts, G. and White, L., 2012. A climate trend analysis of Uganda. 2327-6932, US Geological Survey, Reston, VA, USA.

Gale, L., Harrison, S., Ward, T., O'Donnell, K., Milus, E., Gale, S. and Kistler, H., 2011a. Nivalenol-type populations of *Fusarium graminearum* and *F. asiaticum* are prevalent on wheat in southern Louisiana. Phytopathology 101: 124-134.

Gale, L.R., Harrison, S.A., Ward, T.J., O'Donnell, K., Milus, E.A., Gale, S.W. and Kistler, H.C., 2011b. Nivalenol-type populations of Fusarium graminearum and F. asiaticum are prevalent on wheat in southern Louisiana. Phytopathology 101: 124-134.

Gambacorta, S., Solfrizzo, H., Visconti, A., Powers, S., Cossalter, A., Pinton, P. and Oswald, I., 2013. Validation study on urinary biomarkers of exposure for aflatoxin B1, ochratoxin A, fumonisin B1, deoxynivalenol and zearalenone in piglets. World Mycotoxin Journal 6: 299-308.

Gamble, M.V., Liu, X., Ahsan, H., Pilsner, J.R., Ilievski, V., Slavkovich, V., Parvez, F., Chen, Y., Levy, D., Factor-Litvak, P. and Graziano, J.H., 2006. Folate and arsenic metabolism: a double-blind, placebo-controlled folic acid-supplementation trial in Bangladesh. Am J Clin Nutr 84: 1093-1101.

Gamble, M.V., Liu, X., Ahsan, H., Pilsner, R., Ilievski, V., Slavkovich, V., Parvez, F., Levy, D., Factor-Litvak, P. and Graziano, J.H., 2005. Folate, homocysteine, and arsenic metabolism in arsenic-exposed individuals in Bangladesh. Environ Health Perspect 113: 1683-1688.

Garrido, N., Iha, M., Santos Ortolani, M. and Duarte Fávaro, R., 2003. Occurrence of aflatoxins M1 and M2 in milk commercialized in Ribeirão Preto-SP, Brazil. Food Additives & Contaminants 20: 70-73.

Gething, P.W., Patil, A.P., Smith, D.L., Guerra, C.A., Elyazar, I.R., Johnston, G.L., Tatem, A.J. and Hay, S.I., 2011. A new world malaria map: Plasmodium falciparum endemicity in 2010. Malaria journal 10: 378-393.

Giannitti, F., Diab, S.S., Pacin, A.M., Barrandeguy, M., Larrere, C., Ortega, J. and Uzal, F.A., 2011. Equine leukoencephalomalacia (ELEM) due to fumonisins B1 and B2 in Argentina. Pesquisa Veterinária Brasileira 31: 407-412.

Gong, Y., Cardwell, K., Hounsa, A., Egal, S., Turner, P., Hall, A. and Wild, C., 2002. Dietary aflatoxin exposure and impaired growth in young children from Benin and Togo: cross sectional study. Bmj 325: 20-21.

Gong, Y., Egal, S., Hounsa, A., Turner, P., Hall, A., Cardwell, K. and Wild, C., 2003. Determinants of aflatoxin exposure in young children from Benin and Togo, West Africa: the critical role of weaning. International journal of epidemiology 32: 556-562.

Gong, Y., Hounsa, A., Egal, S., Turner, P.C., Sutcliffe, A.E., Hall, A.J., Cardwell, K. and Wild, C.P., 2004. Postweaning exposure to aflatoxin results in impaired child growth: a longitudinal study in Benin, West Africa. Environ Health Perspect 112: 1334-1338.

Greco, M., Pardo, A. and Pose, G., 2015. Mycotoxigenic fungi and natural co-occurrence of mycotoxins in rainbow trout (Oncorhynchus mykiss) feeds. Toxins 7: 4595-4609.

Groopman, J.D., Egner, P.A., Schulze, K.J., Wu, L.S.-F., Merrill, R., Mehra, S., Shamim, A.A., Ali, H., Shaikh, S. and Gernand, A., 2014. Aflatoxin exposure during the first 1000 days of life in rural South Asia assessed by aflatoxin B 1-lysine albumin biomarkers. Food and Chemical Toxicology 74: 184-189.

Groopman, J.D., Kensler, T.W. and Wild, C.P., 2008. Protective interventions to prevent aflatoxin-induced carcinogenesis in developing countries. Annu. Rev. Public Health 29: 187-203.

Groopman, J.D., Wild, C.P., Hasler, J., Junshi, C., Wogan, G.N. and Kensler, T.W., 1993. Molecular epidemiology of aflatoxin exposures: validation of aflatoxin-N7-guanine levels in urine as a biomarker in experimental rat models and humans. Environ Health Perspect 99: 107-113.

Groopman, J.D., Wogan, G.N., Roebuck, B.D. and Kensler, T.W., 1994. Molecular biomarkers for aflatoxins and their application to human cancer prevention. Cancer research 54: 1907-1911.

Guengerich, F.P., Johnson, W.W., Shimada, T., Ueng, Y.-F., Yamazaki, H. and Langouët, S., 1998. Activation and detoxication of aflatoxin B 1. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 402: 121-128.

Gun, S.Y., Claser, C., Tan, K.S.W. and Rénia, L., 2014. Interferons and interferon regulatory factors in malaria. Mediators of inflammation 2014: 243713.

Hall, M.N., Liu, X., Slavkovich, V., Ilievski, V., Pilsner, J.R., Alam, S., Factor-Litvak, P., Graziano, J.H. and Gamble, M.V., 2009. Folate, Cobalamin, Cysteine, Homocysteine, and Arsenic Metabolism among Children in Bangladesh. Environ Health Perspect 117: 825-831.

Hell, K. and Mutegi, C., 2011. Aflatoxin control and prevention strategies in key crops of Sub-Saharan Africa. 5: 459-466.

Hernandez-Vargas, H., Castelino, J., Silver, M.J., Dominguez-Salas, P., Cros, M.-P., Durand, G., Le Calvez-Kelm, F., Prentice, A.M., Wild, C.P. and Moore, S.E., 2015. Exposure to aflatoxin B1 in utero is associated with DNA methylation in white blood cells of infants in The Gambia. International journal of epidemiology 44: 1238-1248.

Ho, M., Schollaardt, T., Snape, S., Looareesuwan, S., Suntharasamai, P. and White, N.J., 1998. Endogenous interleukin-10 modulates proinflammatory response in Plasmodium falciparum malaria. Journal of Infectious Diseases 178: 520-525.

Hoddinott, J. and Yohannes, Y., 2002. Dietary diversity as a food security indicator, Food consumption and nutrition division discussion paper, pp. 94.

Horn, C.C., 2008. Why is the neurobiology of nausea and vomiting so important? Appetite 50: 430-434.

Hsieh, L.-L. and Hsieh, T.-T., 1993. Detection of aflatoxin B1-DNA adducts in human placenta and cord blood. Cancer research 53: 1278-1280.

Hu, W.-C., 2013. Human immune responses to Plasmodium falciparum infection: molecular evidence for a suboptimal THαβ and TH17 bias over ideal and effective traditional TH1 immune response. Malaria journal 12: 392-410.

Hueza, I.M., Raspantini, P.C.F., Raspantini, L.E.R., Latorre, A.O. and Górniak, S.L., 2014. Zearalenone, an Estrogenic Mycotoxin, Is an Immunotoxic Compound. Toxins 6: 1080-1095.

IARC, 2002a. Fumonisin B. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene.

IARC, 2002b. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC Monogr Eval Carcinog Risks Hum 82: 1-556.

IARC, 2002c. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC Monogr Eval Carcinog Risks Hum 82: 171-300.

Ibanez-Vea, M., Lizarraga, E., Gonzalez-Penas, E. and Lopez de Cerain, A., 2012. Co-occurrence of type-A and type-B trichothecenes in barley from a northern region of Spain. Food Control 25: 81-88.

Ibáñez-Vea, M., Lizarraga, E., González-Peñas, E. and López de Cerain, A., 2012. Co-occurrence of type-A and type-B trichothecenes in barley from a northern region of Spain. Food Control 25: 81-88.

Ibrahim, K. and El-Sayed, E., 2015. Potential role of nutrients on immunity. 23: 464-474.

Inoue, T., Nagatomi, Y., Uyama, A. and Mochizuki, N., 2013. Degradation of aflatoxin B1 during the fermentation of alcoholic beverages. Toxins 5: 1219-1229.

Ishii, K., Maeda, K., Kamataki, T. and Kato, R., 1986. Mutagenic activation of aflatoxin B1 by several forms of purified cytochrome P-450. Mutat Res 174: 85-88.

Jagannathan, P., Eccles-James, I., Bowen, K., Nankya, F., Auma, A., Wamala, S., Ebusu, C., Muhindo, M.K., Arinaitwe, E. and Briggs, J., 2014. IFNγ/IL-10 co-producing cells dominate the CD4 response to malaria in highly exposed children. PLoS Pathog 10: e1003864.

Jagannathan, P., Muhindo, M.K., Kakuru, A., Arinaitwe, E., Greenhouse, B., Tappero, J., Rosenthal, P.J., Kaharuza, F., Kamya, M.R. and Dorsey, G., 2012. Increasing incidence of malaria in children despite insecticide-treated bed nets and prompt anti-malarial therapy in Tororo, Uganda. Malaria journal 11: 435-442.

Jager, A.V., Ramalho, F.S., Zambelli, L.N. and Oliveira, C.A.F., 2011. Biomarkers of aflatoxin exposure and its relationship with the hepatocellular carcinoma, Aflatoxins-Biochemistry and Molecular Biology. InTech.

Jamali, R., Arj, A., Razavizade, M. and Aarabi, M.H., 2016. Prediction of Nonalcoholic Fatty Liver Disease Via a Novel Panel of Serum Adipokines. Medicine 95: e2630.

Jarrett, J.T., 2015. The biosynthesis of thiol-and thioether-containing cofactors and secondary metabolites catalyzed by radical S-adenosylmethionine enzymes. Journal of Biological Chemistry 290: 3972-3979.

JECFA, 2001a. In Safety evaluation of certain mycotoxins in food. Joint WHO/FAO expert committee on food addives. WHO Food Additives series 47: 281-415.

JECFA, 2001b. T-2 and HT-2 toxins. Safety Evaluation of Certain Mycotoxins in Food, Joint Expert Committee on Food Additives (JECFA), WHO Food Additives Series. 47: 557-652.

JECFA, 2010. Evaluation of Certain Food Additives: Seventy-first Report of the Joint FAO/WHO Expert Committee on Food Additives, 956. World Health Organization.

JECFA, 2011. Deoxynivalenol. Safety evaluation of certain contaminants in food: prepared by the Seventy-second meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), Safety evaluation of certain contaminants in food: prepared by the Seventy-second meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). pp. 317-485.

Jiang, M., Peng, X., Fang, J., Cui, H., Yu, Z. and Chen, Z., 2015. Effects of Aflatoxin B1 on T-Cell Subsets and mRNA Expression of Cytokines in the Intestine of Broilers. International journal of molecular sciences 16: 6945-6959.

Jiang, Y., Jolly, P.E., Ellis, W.O., Wang, J.S., Phillips, T.D. and Williams, J.H., 2005. Aflatoxin B1 albumin adduct levels and cellular immune status in Ghanaians. Int Immunol 17: 807-814.

Jiang, Y., Jolly, P.E., Preko, P., Wang, J.-S., Ellis, W.O., Phillips, T.D. and Williams, J.H., 2008. Aflatoxin-related immune dysfunction in health and in human immunodeficiency virus disease. Clinical and Developmental Immunology 2008: 1-12.

Joffe, A., 1974. Toxicity of *Fusarium poae* and *F. Sporotrichioides* and its relation to alimentary toxic aleukia. In: I. Purchase (Ed.), Mycotoxins. Elsevier, Amsterdam, pp. 229-262.

Kaaya, A.N. and Kyamuhangire, W., 2006. The effect of storage time and agroecological zone on mould incidence and aflatoxin contamination of maize from traders in Uganda. International Journal of food microbiology 110: 217-223.

Kaaya, N. and Warren, H., 2005. Review of past and present research on Aflatoxin in Uganda. African Journal of Food, Agriculture, Nutrition and Development 5: 1-19.

Kabak, B., 2009. The fate of mycotoxins during thermal food processing. Journal of the Science of Food and Agriculture 89: 549-554.

Kaiko, G.E., Horvat, J.C., Beagley, K.W. and Hansbro, P.M., 2008. Immunological decision-making: how does the immune system decide to mount a helper T-cell response? Immunology 123: 326-338.

Kang, P., Yao, Y., Yang, J., Shen, B., Zhou, Z. and Pei, F., 2013. An animal model of Kashin–Beck disease induced by a low-nutrition diet and exposure to T-2 toxin. Osteoarthritis and Cartilage 21: 1108-1115.

Karlovsky, P., Suman, M., Berthiller, F., De Meester, J., Eisenbrand, G., Perrin, I., Oswald, I.P., Speijers, G., Chiodini, A. and Recker, T., 2016. Impact of food processing and detoxification treatments on mycotoxin contamination. Mycotoxin research 32: 179-205.

Keller, L., Abrunhosa, L., Keller, K., Rosa, C.A., Cavaglieri, L. and Venâncio, A., 2015. Zearalenone and its derivatives α-zearalenol and β-zearalenol decontamination by Saccharomyces cerevisiae strains isolated from bovine forage. Toxins 7: 3297-3308.

Kemp, K., Akanmori, B., Adabayeri, V., Goka, B., Kurtzhals, J., Behr, C. and Hviid, L., 2002. Cytokine production and apoptosis among T cells from patients under treatment for Plasmodium falciparum malaria. Clinical & Experimental Immunology 127: 151-157.

Kensler, T.W., Egner, P.A., Wang, J.B., Zhu, Y.R., Zhang, B.C., Lu, P.X., Chen, J.G., Qian, G.S., Kuang, S.Y., Jackson, P.E., Gange, S.J., Jacobson, L.P., Munoz, A. and Groopman,

J.D., 2004. Chemoprevention of hepatocellular carcinoma in aflatoxin endemic areas. Gastroenterology 127: 310-318.

Kensler, T.W., Roebuck, B.D., Wogan, G.N. and Groopman, J.D., 2011. Aflatoxin: a 50-year odyssey of mechanistic and translational toxicology. Toxicol Sci 120 Suppl 1: 28-48.

Kew, M.C., 2013. Aflatoxins as a cause of hepatocellular carcinoma. J Gastrointestin Liver Dis 22: 305-310.

Khlangwiset, P., Shephard, G.S. and Wu, F., 2011. Aflatoxins and growth impairment: a review. Crit Rev Toxicol 41: 740-755.

Khlangwiset, P. and Wu, F., 2010. Costs and efficacy of public health interventions to reduce aflatoxin-induced human disease. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 27: 998-1014.

Kilimo Trust, K., 2015. Analysis of the Banana Value Chains in Tanzania and Uganda. Consumption, Productivity and Challenges.

Kimura, M., Kaneko, I., Komiyama, M., Takatsuki, A., Koshino, H., Yoneyama, K. and Yamaguchi, I., 1998. Trichothecene 3-O-Acetyltransferase Protects Both the Producing Organism and Transformed Yeast from Related Mycotoxins. Cloning and Characterization of Tri101. Journal of Biological Chemistry 273: 1654-1661.

Kirk, G.D., Turner, P.C., Gong, Y., Lesi, O.A., Mendy, M., Goedert, J.J., Hall, A.J., Whittle, H., Hainaut, P., Montesano, R. and Wild, C.P., 2005. Hepatocellular carcinoma and polymorphisms in carcinogen-metabolizing and DNA repair enzymes in a population with aflatoxin exposure and hepatitis B virus endemicity. Cancer Epidemiol Biomarkers Prev 14: 373-379.

Kjøllesdal, M.K., Hjellset, V.T. and Høstmark, A.T., 2014. Blood Glucose Measured at Several Time Points and Correlation with Incremental Area under the Curve. Journal of Nutrition & Food Sciences 4: 1-2.

Kongkapan, J., Giorgi, M., Poapolathep, S., Isariyodom, S. and Poapolathep, A., 2016. Toxicokinetics and tissue distribution of nivalenol in broiler chickens. Toxicon 111: 31-36.

Königs, M., Mulac, D., Schwerdt, G., Gekle, M. and Humpf, H.-U., 2009. Metabolism and cytotoxic effects of T-2 toxin and its metabolites on human cells in primary culture. Toxicology 258: 106-115.

Kosawang, C., Karlsson, M., Jensen, D., Kilokpimol, A. and Collinge, D., 2014. Transcriptomic profiling to identify genes involved in *Fusarium* mycotoxin deoxynivalenol and zearalenone tolerance in the mycoparasitic fungus *Clonostachys rosea*, BMC Genomics, pp. 55.

Kőszegi, T. and Poór, M., 2016. Ochratoxin A: molecular interactions, mechanisms of toxicity and prevention at the molecular level. Toxins 8: 111-135.

Kovac, A.L., 2016. Mechanisms of nausea and vomiting. Postoperative Nausea and Vomiting: 13.

Krska, R., Malachova, A., Berthiller, F. and van Egmond, H., 2014. Determination of T-2 and HT-2 toxins in food and feed: an update. World Mycotoxin J 7: 131-142.

Kuca, K. and Pohanka, M., 2010. Chemical warfare agents. Mol Clin Environ Toxicol 100: 543-559.

Kumi, J., Mitchell, N., Asare, G., Dotse, E., Kwaa, F., Phillips, T. and Ankrah, N., 2014. Aflatoxins and fumonisins contamination of home-made food (weanimix) from cereal-legume blends for children. Ghana medical journal 48: 121-126.

Laishram, D.D., Sutton, P.L., Nanda, N., Sharma, V.L., Sobti, R.C., Carlton, J.M. and Joshi, H., 2012. The complexities of malaria disease manifestations with a focus on asymptomatic malaria. Malaria journal 11: 29-43.

Lang, I., 1999. Noxious stimulation of emesis. Digestive diseases and sciences 44: 58-63.

Larypoor, M., Bayat, M., Zuhair, M.H., Sepahy, A.A. and Amanlou, M., 2013. Evaluation of The Number of CD4+ CD25+ FoxP3+ Treg Cells in Normal Mice Exposed to AFB1 and Treated with Aged Garlic Extract. Cell Journal (Yakhteh) 15: 37-44.

Le Floch, J.-P., Escuyer, P., Baudin, E., Baudon, D. and Perlemuter, L., 1990. Blood glucose area under the curve: methodological aspects. Diabetes care 13: 172-175.

Lechenet, M., Bretagnolle, V., Bockstaller, C., Boissinot, F., Petit, M.S., Petit, S. and Munier-Jolain, N.M., 2014. Reconciling pesticide reduction with economic and environmental sustainability in arable farming. PLoS One 9: e97922.

Li, Y., Burns, K.A., Arao, Y., Luh, C.J. and Korach, K.S., 2012. Differential estrogenic actions of endocrine-disrupting chemicals bisphenol A, bisphenol AF, and zearalenone through estrogen receptor α and β in vitro. Environ Health Perspect 120: 1029-1035.

Li, Y., Zhang, B., Huang, K., He, X., Luo, Y., Liang, R., Luo, H., Shen, X.L. and Xu, W., 2014. Mitochondrial proteomic analysis reveals the molecular mechanisms underlying reproductive toxicity of zearalenone in MLTC-1 cells. Toxicology 324: 55-67.

Lingwood, R.J., Boyle, P., Milburn, A., Ngoma, T., Arbuthnott, J., McCaffrey, R., Kerr, S.H. and Kerr, D.J., 2008. The challenge of cancer control in Africa. Nature reviews cancer 8: 398-403.

Liu, L. and Massey, T.E., 1992. Bioactivation of aflatoxin B1 by lipoxygenases, prostaglandin H synthase and cytochrome P450 monooxygenase in guinea-pig tissues. Carcinogenesis 13: 533-539.

Liu, Y., Chang, C.-C.H., Marsh, G.M. and Wu, F., 2012. Population attributable risk of aflatoxin-related liver cancer: systematic review and meta-analysis. European journal of cancer 48: 2125-2136.

Liu, Y. and Wu, F., 2010. Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. Environ Health Perspect 118: 818-824.

Long, M., Zhang, Y., Li, P., Yang, S.-H., Zhang, W.-K., Han, J.-X., Wang, Y. and He, J.-B., 2016. Intervention of grape seed proanthocyanidin extract on the subchronic immune injury in mice induced by aflatoxin B1. International journal of molecular sciences 17: 516-525.

Lunn, R.M., Zhang, Y.-J., Wang, L.-Y., Chen, C.-J., Lee, P.-H., Lee, C.-S., Tsai, W.-Y. and Santella, R.M., 1997. P53 mutations, chronic hepatitis b virus infection, and alfatoxin exposure in hepatocellular carcinoma in Taiwan. Cancer research 57: 3471-3477.

MacGillivray, D.M. and Kollmann, T.R., 2014. The role of environmental factors in modulating immune responses in early life. Frontiers in immunology 5: 434-445.

Mackroth, M.S., Abel, A., Steeg, C., zur Wiesch, J.S. and Jacobs, T., 2016. Acute Malaria Induces PD1+ CTLA4+ Effector T Cells with Cell-Extrinsic Suppressor Function. PLoS Pathog 12: e1005909.

Malachova, A., van Egmond, H., Berthiller, F. and Krska, R., 2014a. Determination of nivalenol in food and feed: an update. World Mycotoxin Journal 7: 247-255.

Malachova, A., van Egmond, H., Berthiller, F. and Krska, R., 2014b. Determination of nivalenol in food and feed: an update. World Mycotoxin J 7: 247-255.

Male, D., Mitchell, N., Wu, W., Bursian, S., Pestka, J. and Wu, F., 2015. Modelling the anorectic potencies of food-borne trichothecenes by benchmark dose and incremental area under the curve methodology. World Mycotoxin Journal 9: 279-288.

Male, D., Wu, W., Mitchell, N.J., Bursian, S., Pestka, J.J. and Wu, F., 2016. Modeling the emetic potencies of food-borne trichothecenes by benchmark dose methodology. Food and Chemical Toxicology 94: 178-185.

Malir, F., Ostry, V., Pfohl-Leszkowicz, A., Malir, J. and Toman, J., 2016. Ochratoxin A: 50 years of research. Toxins 8: 191-239.

Marusteri, M. and Bacarea, V., 2010. Comparing groups for statistical differences: how to choose the right statistical test? Biochemia medica 20: 15-32.

May, J., Lell, B., Luty, A.J., Meyer, C.G. and Kremsner, P.G., 2000. Plasma Interleukin-10: Tumor Necrosis Factor (TNF)—α Ratio Is Associated with TNF Promoter Variants and Predicts Malarial Complications. Journal of Infectious Diseases 182: 1570-1573.

Mbengue, B., Niang, B., Niang, M.S., Varela, M.L., Fall, B., Fall, M.M., Diallo, R.N., Diatta, B., Gowda, D. and Dieye, A., 2015. Inflammatory cytokine and humoral responses to Plasmodium falciparum glycosylphosphatidylinositols correlates with malaria immunity and pathogenesis. Immunity, Inflammation and Disease 4: 24-34.

McCormick, S.P., 2013. Microbial detoxification of mycotoxins. Journal of chemical ecology 39: 907-918.

McCormick, S.P., Stanley, A.M., Stover, N.A. and Alexander, N.J., 2011. Trichothecenes: from simple to complex mycotoxins. Toxins 3: 802-814.

McCoy, K.D. and Le Gros, G., 1999. The role of CTLA-4 in the regulation of T cell immune responses. Immunology and cell biology 77: 1-10.

McCoy, L.F., Scholl, P.F., Schleicher, R.L., Groopman, J.D., Powers, C.D. and Pfeiffer, C.M., 2005. Analysis of aflatoxin B1-lysine adduct in serum using isotope-dilution liquid chromatography/tandem mass spectrometry. Rapid communications in mass spectrometry 19: 2203-2210.

McCoy, L.F., Scholl, P.F., Sutcliffe, A.E., Kieszak, S.M., Powers, C.D., Rogers, H.S., Gong, Y.Y., Groopman, J.D., Wild, C.P. and Schleicher, R.L., 2008. Human aflatoxin albumin adducts quantitatively compared by ELISA, HPLC with fluorescence detection, and HPLC with isotope dilution mass spectrometry. Cancer Epidemiology Biomarkers & Prevention 17: 1653-1657.

McDonald, J.H., 2009. Handbook of biological statistics, 2. Sparky House Publishing Baltimore, MD, Baltimore, Maryland, USA.

Meissonnier, G.M., Pinton, P., Laffitte, J., Cossalter, A.-M., Gong, Y.Y., Wild, C.P., Bertin, G., Galtier, P. and Oswald, I.P., 2008. Immunotoxicity of aflatoxin B1: impairment of the cell-mediated response to vaccine antigen and modulation of cytokine expression. Toxicology and applied pharmacology 231: 142-149.

Mitchell, N.J., Hsu, H.-H., Chandyo, R.K., Shrestha, B., Bodhidatta, L., Tu, Y.-K., Gong, Y.-Y., Egner, P.A., Ulak, M. and Groopman, J.D., 2017. Aflatoxin exposure during the first 36 months of life was not associated with impaired growth in Nepalese children: An extension of the MAL-ED study. PLoS One 12: e0172124.

Mitchell, N.J., Riley, R.T., Egner, P.A., Groopman, J.D. and Wu, F., 2016. Chronic aflatoxin exposure in children living in Bhaktapur, Nepal: Extension of the MAL-ED study. Journal of Exposure Science and Environmental Epidemiology 27: 106-111.

Mohsenzadeh, M.S., Hedayati, N., Riahi-Zanjani, B. and Karimi, G., 2016. Immunosuppression following dietary aflatoxin B1 exposure: a review of the existing evidence. Toxin Reviews 35: 121-127.

Moursi, M.M., Arimond, M., Dewey, K.G., Trèche, S., Ruel, M.T. and Delpeuch, F., 2008. Dietary diversity is a good predictor of the micronutrient density of the diet of 6-to 23-month-old children in Madagascar. J Nutr 138: 2448-2453.

Mupunga, I., Izaaks, C.D., Shai, L.J. and Katerere, D.R., 2016. Aflatoxin biomarkers in hair may facilitate long-term exposure studies. Journal of Applied Toxicology 37: 395-399.

Muro-Cach, C.A., Stedeford, T., Banasik, M., Suchecki, T.T. and Persad, A.S., 2004. Mycotoxins: Mechanisms of toxicity and methods of detection for identifying exposed individuals. Journal of Land Use & Environmental Law: 537-556.

Nagao, T., Golor, G., Hagenmaier, H. and Neubert, D., 1993. Teratogenic potency of 2, 3, 4, 7, 8-pentachlorodibenzofuran and of three mixtures of polychlorinated dibenzo-p-dioxins and dibenzofurans in mice. Problems with risk assessment using TCDD toxic-equivalency factors. Archives of toxicology 67: 591-597.

Nankumbi, J. and Muliira, J.K., 2015. Barriers to infant and child-feeding practices: a qualitative study of primary caregivers in Rural Uganda. Journal of health, population, and nutrition 33: 106-116.

Naujokas, M.F., Anderson, B., Ahsan, H., Aposhian, H.V., Graziano, J.H., Thompson, C. and Suk, W.A., 2013. The broad scope of health effects from chronic arsenic exposure: update on a worldwide public health problem. Environmental Health Perspectives (Online) 121: 295-302.

Niikura, M., Inoue, S.-I. and Kobayashi, F., 2011. Role of interleukin-10 in malaria: focusing on coinfection with lethal and nonlethal murine malaria parasites. BioMed Research International 2011: e383962.

Njobeh, P.B., Dutton, M.F., Koch, S.H., Chuturgoon, A., Stoev, S. and Seifert, K., 2009. Contamination with storage fungi of human food from Cameroon. Int J Food Microbiol 135: 193-198.

Nordberg, G.F., 2010. Biomarkers of exposure, effects and susceptibility in humans and their application in studies of interactions among metals in China. Toxicology Letters 192: 45-49.

Nyirenda, T.S., Molyneux, M.E., Kenefeck, R., Walker, L.S., MacLennan, C.A., Heyderman, R.S. and Mandala, W.L., 2015. T-regulatory cells and inflammatory and inhibitory cytokines in Malawian children residing in an area of high and an area of low malaria transmission during acute uncomplicated malaria and in convalescence. Journal of the Pediatric Infectious Diseases Society 4: 232-241.

Nzwalo, H. and Cliff, J., 2011. Konzo: from poverty, cassava, and cyanogen intake to toxico-nutritional neurological disease. PLoS Negl Trop Dis 5: e1051.

Obade, M.I., Andang'o, P., Obonyo, C. and Lusweti, F., 2015. Exposure of children 4 to 6 months of age to aflatoxin in Kisumu County, Kenya. African Journal of Food, Agriculture, Nutrition and Development 15: 9949-9963.

Othoro, C., Lal, A.A., Nahlen, B., Koech, D., Orago, A.S. and Udhayakumar, V., 1999. A low interleukin-10 tumor necrosis factor-α ratio is associated with malaria anemia in children residing in a holoendemic malaria region in western Kenya. Journal of Infectious Diseases 179: 279-282.

Partanen, H.A., El-Nezami, H.S., Leppänen, J.M., Myllynen, P.K., Woodhouse, H.J. and Vähäkangas, K.H., 2009. Aflatoxin B1 transfer and metabolism in human placenta. Toxicological Sciences 113: 216-225.

Pascual-Ahuir, A., Vanacloig-Pedros, E. and Proft, M., 2014. Toxicity mechanisms of the food contaminant citrinin: application of a quantitative yeast model. Nutrients 6: 2077-2087.

Perera, M., Herath, N., Pathirana, S., Phone-Kyaw, M., Alles, H., Mendis, K., Premawansa, S. and Handunnetti, S., 2013. Association of high plasma TNF-alpha levels and TNF-alpha/IL-10 ratios with TNF2 allele in severe P. falciparum malaria patients in Sri Lanka. Pathogens and global health 107: 21-29.

Perez-Mazliah, D. and Langhorne, J., 2015. CD4T-cell subsets in malaria: TH1/TH2 revisited. CD4+ T cell differentiation in infection: amendments to the Th1/Th2 axiom 5: 671-678.

Pestka, J., 2010a. Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. Arch Toxicol 84: 663-679.

Pestka, J., 2010b. Toxicological mechanisms and potential health effects of deoxynivalenol and nivalenol. World Mycotoxin Journal 3: 323-347.

Pestka, J.J., 2010c. Deoxynivalenol-induced proinflammatory gene expression: Mechanisms and pathological sequelae. Toxins 2: 1300-1317.

Pestka, J.J., 2010d. Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. Archives of toxicology 84: 663-679.

Pestka, J.J. and Smolinski, A.T., 2005. Deoxynivalenol: toxicology and potential effects on humans. Journal of Toxicology and Environmental Health, Part B 8: 39-69.

Pestka, J.J., Zhou, H.-R., Moon, Y. and Chung, Y., 2004. Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unraveling a paradox. Toxicology Letters 153: 61-73.

Peyron, F., Burdin, N., Ringwald, P., Vuillez, J., Rousset, F. and Banchereau, J., 1994. High levels of circulating IL-10 in human malaria. Clinical & Experimental Immunology 95: 300-303.

Pfeiffer, E., Kommer, A., Dempe, J.S., Hildebrand, A.A. and Metzler, M., 2011. Absorption and metabolism of the mycotoxin zearalenone and the growth promotor zeranol in Caco-2 cells in vitro. Molecular nutrition & food research 55: 560-567.

Pfohl-Leszkowicz, A., 2009. Ochratoxin A and aristolochic acid involvement in nephropathies and associated urothelial tract tumours. Arhiv za higijenu rada i toksikologiju 60: 465-482.

Phillips, P., Henry, G., Graffham, A., Vilpoux, O., Titapiwatanakun, B. and Taylor, D., 1999. Global Cassava Market Study: Business Opportunities for the Use of Cassava. Assembled by dTp Studies Inc. and funded by IDRC, Ottawa, and IFAD, Rome.

Pilsner, J.R., Liu, X., Ahsan, H., Ilievski, V., Slavkovich, V., Levy, D., Factor-Litvak, P., Graziano, J.H. and Gamble, M.V., 2009. Folate deficiency, hyperhomocysteinemia, low urinary creatinine, and hypomethylation of leukocyte DNA are risk factors for arsenic-induced skin lesions. Environ Health Perspect 117: 254-260.

Pinton, P., Guzylack-Piriou, L., Kolf-Clauw, M. and P Oswald, I., 2012a. The effect on the intestine of some fungal toxins: The trichothecenes. Current Immunology Reviews 8: 193-208.

Pinton, P., Tsybulskyy, D., Lucioli, J., Laffitte, J., Callu, P., Lyazhri, F., Grosjean, F., Bracarense, A.-P., Martine, K.-C. and Oswald, I.P., 2012b. Toxicity of deoxynivalenol and its acetylated derivatives on the intestine: differential effects on morphology, barrier function, tight junctions proteins and MAPKinases. Toxicological Sciences 130: 180-190.

Poapolathep, A., Sugita-Konishi, Y., Doi, K. and Kumagai, S., 2003. The fates of trichothecene mycotoxins, nivalenol and fusarenon-X, in mice. Toxicon 41: 1047-1054.

Pralatnet, S., Poapolathep, S., Imsilp, K., Tanhan, P., Isariyodom, S., Kumagai, S. and Poapolathep, A., 2015. The fate and tissue disposition of deoxynivalenol in broiler chickens. The Journal of Veterinary Medical Science 77: 1151-1155.

Prasad, A.S., 2013. Discovery of human zinc deficiency: its impact on human health and disease. Advances in Nutrition: An International Review Journal 4: 176-190.

Prelusky, D.B. and Trenholm, H.L., 1993. The efficacy of various classes of anti-emetics in preventing deoxynivalenol-induced vomiting in swine. Natural toxins 1: 296-302.

Pronk, E.J., Schothorst, R.C. and van Egmond, H.P., 2002. Toxicology and Occurrence of Nivalenol, Fusarenon X, Diacetoxyscirpenol, Neosolaniol and 3- and 15-acetyldeoxynivalenol: a Review of Six Trichothecenes. RIVM, 75 pp.

Qian, G.-S., Ross, R.K., Yu, M.C., Yuan, J.-M., Gao, Y.-T., Henderson, B.E., Wogan, G.N. and Groopman, J.D., 1994. A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China. Cancer Epidemiology and Prevention Biomarkers 3: 3-10.

Qian, G., Tang, L., Guo, X., Wang, F., Massey, M.E., Su, J., Guo, T.L., Williams, J.H., Phillips, T.D. and Wang, J.S., 2014. Aflatoxin B1 modulates the expression of phenotypic markers and cytokines by splenic lymphocytes of male F344 rats. Journal of Applied Toxicology 34: 241-249.

Quiles, J.M., Manyes, L., Luciano, F., Manes, J. and Meca, G., 2015. Influence of the antimicrobial compound allyl isothiocyanate against the Aspergillus parasiticus growth and its aflatoxins production in pizza crust. Food and Chemical Toxicology 83: 222-228.

Raisuddin, S., Singh, K., Zaidi, S., Paul, B. and Ray, P., 1993. Immunosuppressive effects of aflatoxin in growing rats. Mycopathologia 124: 189-194.

Ramos, A. and Hernandez, E., 1996. In situ absorption of aflatoxins in rat small intestine. Mycopathologia 134: 27-30.

Raters, M. and Matissek, R., 2008. Thermal stability of aflatoxin B1 and ochratoxin A. Mycotoxin research 24: 130-134.

Reddy, L. and Bhoola, K., 2010. Ochratoxins—Food contaminants: Impact on human health. Toxins 2: 771-779.

Richard, J., Payne, G., eds, Desjardins, A., Maragos, C., Norred, W. and Pestka, J., 2003. Mycotoxins: risks in plant, animal and human systems. CAST Task Force Report 139: 101-103.

Ross, R.K., Yu, M., Henderson, B., Yuan, J.-M., Qian, G.-S., Tu, J.-T., Gao, Y.-T., Wogan, G. and Groopman, J.D., 1992. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. The Lancet 339: 943-946.

Ruel, M.T. and Menon, P., 2002. Child feeding practices are associated with child nutritional status in Latin America: innovative uses of the demographic and health surveys. J Nutr 132: 1180-1187.

Rwabwogo, M.O., 2007. Uganda districts information handbook. Fountain Publishers.

Rwashana, A., Nakubulwa, S., Nakakeeto-Kijjambu, M., Adam, T., Kalibala, S., Schenk, K., Weiss, D., Elson, L., Fosu, G. and Salin, M., 2015. Uganda Malaria Indicator Survey 2014-15: Key indicators. Health Research Policy and Systems 12: 1-139.

Saengtienchai, T., Poapolathep, S., Isariyodom, S., Ikenaka, Y., Ishizuka, M. and Poapolathep, A., 2014. Toxicokinetics and tissue depletion of Fusarenon-X and its metabolite nivalenol in piglets. Food Chem Toxicol 66: 307-312.

Safe, S.H., 1998. Hazard and risk assessment of chemical mixtures using the toxic equivalency factor approach. Environ Health Perspect 106: 1051-1058.

SCF, 2002. Opinion of the Scientific Committee on Food on Fusarium toxins. Part 6: Group evaluation of T-2 toxin, HT-2 toxin, nivalenol and deoxynivalenol. In: E.C. European Commission (Ed.), SCF/CS/CNTM/MYC/27.

Scholl, P., Musser, S.M., Kensler, T.W. and Groopman, J.D., 1995. Molecular biomarkers for aflatoxins and their application to human liver cancer. Pharmacogenetics and Genomics 5: S171-S176.

Scholl, P.F. and Groopman, J.D., 2008. Long-term stability of human aflatoxin B1 albumin adducts assessed by isotope dilution mass spectrometry and high-performance liquid chromatography–fluorescence. Cancer Epidemiology Biomarkers & Prevention 17: 1436-1439.

Scholl, P.F., Turner, P.C., Sutcliffe, A.E., Sylla, A., Diallo, M.S., Friesen, M.D., Groopman, J.D. and Wild, C.P., 2006. Quantitative comparison of aflatoxin B1 serum albumin adducts in humans by isotope dilution mass spectrometry and ELISA. Cancer Epidemiology Biomarkers & Prevention 15: 823-826.

Scott, P., 2012. Recent research on fumonisins: a review. Food additives & contaminants: part A 29: 242-248.

Šegvić Klarić, M., 2012. Adverse Effects Of Combined Mycotoxins/Štetni Učinci Kombiniranih Mikotoksina. Archives of Industrial Hygiene and Toxicology 63: 519-530.

Shank, R.A., Foroud, N.A., Hazendonk, P., Eudes, F. and Blackwell, B.A., 2011. Current and future experimental strategies for structural analysis of trichothecene mycotoxins—a prospectus. Toxins 3: 1518-1553.

Shively, G. and Hao, J., 2012. A review of agriculture, food security and human nutrition issues in Uganda. Department of Agricultural Economics, Purdue University 12: 1-28.

Shively, G.E., 2017. Infrastructure mitigates the sensitivity of child growth to local agriculture and rainfall in Nepal and Uganda. Proceedings of the National Academy of Sciences 114: 903-908.

Silva-Filho, J., Caruso-Neves, C. and Pinheiro, A., 2014. IL-4: an important cytokine in determining the fate of T cells. Biophysical Reviews 6: 111-118.

Simsek, S., Ovando-Martinez, M., Ozsisli, B., Whitney, K. and Ohm, J.-B., 2013. Occurrence of deoxynivalenol and deoxynivalenol-3-glucoside in hard red spring wheat grown in the USA. Toxins 5: 2656-2670.

Sinnis, P. and Zavala, F., 2008. The skin stage of malaria infection: biology and relevance to the malaria vaccine effort. 3: 275-278.

Sintov, A., Bialer, M. and Yagen, B., 1986. Pharmacokinetics of T-2 toxin and its metabolite HT-2 toxin, after intravenous administration in dogs. Drug metabolism and disposition 14: 250-254.

Smela, M.E., Currier, S.S., Bailey, E.A. and Essigmann, J.M., 2001. The chemistry and biology of aflatoxin B1: from mutational spectrometry to carcinogenesis. Carcinogenesis 22: 535-545.

Smith, A.H. and Steinmaus, C.M., 2009. Health effects of arsenic and chromium in drinking water: recent human findings. Annual review of public health 30: 107-122.

Smith, L.E., Prendergast, A.J., Turner, P.C., Mbuya, M.N.N., Mutasa, K., Kembo, G. and Stoltzfus, R.J., 2015. The Potential Role of Mycotoxins as a Contributor to Stunting in the SHINE Trial. Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America 61: S733-S737.

Smith, M.-C., Madec, S., Coton, E. and Hymery, N., 2016. Natural co-occurrence of mycotoxins in foods and feeds and their in vitro combined toxicological effects. Toxins 8: 94-129.

Sobrova, P., Adam, V., Vasatkova, A., Beklova, M., Zeman, L. and Kizek, R., 2010. Deoxynivalenol and its toxicity. Interdisciplinary toxicology 3: 94-99.

Stadler, M., Bardiau, F., Seidel, L., Albert, A. and Boogaerts, J.G., 2003. Difference in risk factors for postoperative nausea and vomiting. The Journal of the American Society of Anesthesiologists 98: 46-52.

Stanciu, O., Banc, R., Cozma, A., Filip, L., Miere, D., Mañes, J. and Loghin, F., 2015. Occurence of Fusarium Mycotoxins in Wheat from Europe–A Review. Acta Universitatis Cibiniensis. Series E: Food Technology 19: 35-60.

Steinberg, E.B., Henderson, A., Karpati, A., Hoekstra, M., Marano, N., Souza, J.M., Simons, M., Kruger, K., Giroux, J. and Rogers, H.S., 2006. Mysterious outbreaks of gastrointestinal illness associated with burritos supplied through school lunch programs. Journal of Food Protection® 69: 1690-1698.

Steinmaus, C., Carrigan, K., Kalman, D., Atallah, R., Yuan, Y. and Smith, A.H., 2005. Dietary intake and arsenic methylation in a US population. Environ Health Perspect: 1153-1159.

Stenglein, S., 2009. Fusarium poae: a pathogen that needs more attention. Journal of Plant Pathology: 25-36.

Strimbu, K. and Tavel, J.A., 2010. What are biomarkers? Current Opinion in HIV and AIDS 5: 463.

Strosnider, H., Azziz-Baumgartner, E., Banziger, M., Bhat, R.V., Breiman, R., Brune, M.-N., DeCock, K., Dilley, A., Groopman, J. and Hell, K., 2006. Workgroup report: public health strategies for reducing aflatoxin exposure in developing countries. Environ Health Perspect: 1898-1903.

Supriya, C., Akhila, B., Pratap Reddy, K., Girish, B. and Sreenivasula Reddy, P., 2016. Effects of maternal exposure to aflatoxin B1 during pregnancy on fertility output of dams and

developmental, behavioral and reproductive consequences in female offspring using a rat model. Toxicology mechanisms and methods 26: 202-210.

Tajima, O., Schoen, E., Feron, V. and Groten, J., 2002. Statistically designed experiments in a tiered approach to screen mixtures of Fusarium mycotoxins for possible interactions. Food and Chemical Toxicology 40: 685-695.

Thuvander, A., Wikman, C. and Gadhasson, I., 1999. In vitro exposure of human lymphocytes to trichothecenes: individual variation in sensitivity and effects of combined exposure on lymphocyte function. Food and Chemical Toxicology 37: 639-648.

Tor-Agbidye, J., Palmer, V.S., Lasarev, M.R., Craig, A.M., Blythe, L.L., Sabri, M.I. and Spencer, P.S., 1999. Bioactivation of cyanide to cyanate in sulfur amino acid deficiency: relevance to neurological disease in humans subsisting on cassava. Toxicological Sciences 50: 228-235.

Torre, D., Speranza, F., Giola, M., Matteelli, A., Tambini, R. and Biondi, G., 2002. Role of Th1 and Th2 cytokines in immune response to uncomplicated Plasmodium falciparum malaria. Clinical and diagnostic laboratory immunology 9: 348-351.

Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent, J. and Jemal, A., 2015. Global cancer statistics, 2012. CA: a cancer journal for clinicians 65: 87-108.

Tshala-Katumbay, D., Eeg-Olofsson, K.E., Kazadi-Kayembe, T., Tylleskär, T. and Fällmar, P., 2002. Analysis of motor pathway involvement in konzo using transcranial electrical and magnetic stimulation. Muscle & nerve 25: 230-235.

Tuck, M.K., Chan, D.W., Chia, D., Godwin, A.K., Grizzle, W.E., Krueger, K.E., Rom, W., Sanda, M., Sorbara, L. and Stass, S., 2008. Standard operating procedures for serum and plasma collection: early detection research network consensus statement standard operating procedure integration working group. Journal of proteome research 8: 113-117.

Turner, P.C., 2013. The molecular epidemiology of chronic aflatoxin driven impaired child growth. Scientifica 2013: 1-21.

Turner, P.C., Collinson, A.C., Cheung, Y.B., Gong, Y., Hall, A.J., Prentice, A.M. and Wild, C.P., 2007. Aflatoxin exposure in utero causes growth faltering in Gambian infants. International journal of epidemiology 36: 1119-1125.

Turner, P.C., Moore, S.E., Hall, A.J., Prentice, A.M. and Wild, C.P., 2003. Modification of immune function through exposure to dietary aflatoxin in Gambian children. Environ Health Perspect 111: 217-220.

Tylleskär, T., Howlett, W., Rwiza, H., Aquilonius, S., Stålberg, E., Linden, B., Mandahl, A., Larsen, H., Brubaker, G. and Rosling, H., 1993. Konzo: a distinct disease entity with selective upper motor neuron damage. Journal of Neurology, Neurosurgery & Psychiatry 56: 638-643.

Udomkun, P., Wiredu, A.N., Nagle, M., Müller, J., Vanlauwe, B. and Bandyopadhyay, R., 2017. Innovative technologies to manage aflatoxins in foods and feeds and the profitability of application–A review. Food Control 76: 127-138.

Ueno, Y., 1977. Mode of action of trichothecenes. Pure and Applied Chemistry 49: 1737-1745.

Valencia-Quintana, R., Sánchez-Alarcón, J., Tenorio-Arvide, M.G., Deng, Y., Montiel-González, J.M., Gómez-Arroyo, S., Villalobos-Pietrini, R., Cortés-Eslava, J., Flores-Márquez, A.R. and Arenas-Huertero, F., 2015. The microRNAs as potential biomarkers for predicting the onset of aflatoxin exposure in human beings: a review. Global health issues of aflatoxins in food and agriculture: Challenges and opportunities: 102-115.

Van den Berg, M., Birnbaum, L., Bosveld, A., Brunstrom, B., Cook, P. and Feeley, M., 1998a. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ Health Perspect 106: 775-792.

Van den Berg, M., Birnbaum, L., Bosveld, A., Brunström, B., Cook, P., Feeley, M., Giesy, J.P., Hanberg, A., Hasegawa, R. and Kennedy, S.W., 1998b. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ Health Perspect 106: 775-792.

Van den Berg, M., Birnbaum, L., Denison, M., De Vito, M., Farland, W., Feeley, M., Fiedler, H., Hakansson, H., Hanberg, A., Haws, L., Rose, M., Safe, S., Schrenk, D., Tohyama, C., Tritscher, A., Tuomisto, J., Tysklind, M., Walker, N. and Peterson, R., 2006a. The 2005 World Health Organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicol Sci 93: 223-241.

Van den Berg, M., Birnbaum, L.S., Denison, M., De Vito, M., Farland, W., Feeley, M., Fiedler, H., Hakansson, H., Hanberg, A. and Haws, L., 2006b. The 2005 World Health Organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicological Sciences 93: 223-241.

van der Lee, T., Zhang, H., van Diepeningen, A. and Waalwijk, C., 2015. Biogeography of Fusarium graminearum species complex and chemotypes: a review. Food additives & contaminants: part A 32: 453-460.

Van Egmond, H.P., Schothorst, R.C. and Jonker, M.A., 2007. Regulations relating to mycotoxins in food. Analytical and Bioanalytical Chemistry 389: 147-157.

Veršilovskis, A., Geys, J., Huybrechts, B., Goossens, E., De Saeger, S. and Callebaut, A., 2012. Simultaneous determination of masked forms of deoxynivalenol and zearalenone after oral dosing in rats by LC-MS/MS. World Mycotoxin Journal 5: 303-318.

Visconti, A., Minervini, F., Lucivero, G. and Gambatesa, V., 1991. Cytotoxic and immunotoxic effects of Fusarium mycotoxins using a rapid colorimetric bioassay. Mycopathologia 113: 181-186.

Wang, Y., Wang, L., Liu, F., Wang, Q., Selvaraj, J.N., Xing, F., Zhao, Y. and Liu, Y., 2016. Ochratoxin A producing fungi, biosynthetic pathway and regulatory mechanisms. Toxins 8: 83-97.

Watson, S., Diedhiou, P., Atehnkeng, J., Dem, A., Bandyopadhyay, R., Srey, C., Routledge, M. and Gong, Y., 2015. Seasonal and geographical differences in aflatoxin exposures in Senegal. World Mycotoxin Journal 8: 525-531.

Weidner, M., Huwel, S., Ebert, F., Schwerdtle, T., Galla, H.-J. and Humpf, H.-U., 2013. Influence of T-2 and HT-2 toxin on the blood-brain barrier in vitro: new experimental hints for neurotoxic effects. PloS one 8: e60484.

WHO, 1993. Biomarkers and risk assessment: concepts and principles. World Health Organization, Geneva.

WHO, 2009. Global health risks: mortality and burden of disease attributable to selected major risks. World Health Organization.

WHO Multicentre Growth Reference Study Group, W.H.O., 2006. WHO child growth standards: length/height for age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age, methods and development. World Health Organization.

Wild, C. and Turner, P., 2002. The toxicology of aflatoxins as a basis for public health decisions. Mutagenesis 17: 471-481.

Wild, C.P. and Gong, Y.Y., 2010. Mycotoxins and human disease: a largely ignored global health issue. Carcinogenesis 31: 71-82.

Williams, J.H., Phillips, T.D., Jolly, P.E., Stiles, J.K., Jolly, C.M. and Aggarwal, D., 2004. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. Am J Clin Nutr 80: 1106-1122.

Wogan, G.N., Kensler, T.W. and Groopman, J.D., 2012. Present and future directions of translational research on aflatoxin and hepatocellular carcinoma. A review. Food additives & contaminants: part A 29: 249-257.

Wolever, T.M., Jenkins, D.J., Jenkins, A.L. and Josse, R.G., 1991. The glycemic index: methodology and clinical implications. Am J Clin Nutr 54: 846-854.

Wortmann, C.S. and Eledu, C.S., 1999. Uganda's agroecological zones: a guide for planners and policy markers 1.

Wu, F., Groopman, J. and Pestka, J., 2014a. Public health impacts of foodborne mycotoxins. Annu Rev Food Sci Technol 5: 351-372.

Wu, F., Groopman, J.D. and Pestka, J.J., 2014b. Public health impacts of foodborne mycotoxins. Annual review of food science and technology 5: 351-372.

Wu, F. and Guclu, H., 2012. Aflatoxin regulations in a network of global maize trade. PLoS One 7: e45151.

Wu, F., Mitchell, N.J., Male, D. and Kensler, T.W., 2014c. Reduced Foodborne Toxin Exposure is a Benefit of Improving Dietary Diversity. Toxicological Sciences 141: 329-334.

Wu, F., Stacy, S.L. and Kensler, T.W., 2013a. Global risk assessment of aflatoxins in maize and peanuts: are regulatory standards adequately protective? Toxicological Sciences 135: 251-259.

Wu, H.-C. and Santella, R., 2012. The role of aflatoxins in hepatocellular carcinoma. Hepatitis monthly 12.

Wu, H.-C., Wang, Q., Wang, L.-W., Yang, H.-I., Ahsan, H., Tsai, W.-Y., Wang, L.-Y., Chen, S.-Y., Chen, C.-J. and Santella, R.M., 2007. Urinary 8-oxodeoxyguanosine, aflatoxin B1 exposure and hepatitis B virus infection and hepatocellular carcinoma in Taiwan. Carcinogenesis 28: 995-999.

Wu, H.-C., Wang, Q., Yang, H.-I., Tsai, W.-Y., Chen, C.-J. and Santella, R.M., 2013b. Global DNA methylation in a population with aflatoxin B1 exposure. Epigenetics 8: 962-969.

Wu, Q., Dohnal, V., Huang, L., Kuca, K. and Yuan, Z., 2010. Metabolic pathways of trichothecenes. Drug Metab Rev 42: 250-267.

Wu, Q., Dohnal, V., Kuca, K. and Yuan, Z., 2013c. Trichothecenes: structure-toxic activity relationships. Curr Drug Metab 14: 641-660.

Wu, Q., Dohnal, V., Kuca, K. and Yuan, Z., 2013d. Trichothecenes: structure-toxic activity relationships. Current drug metabolism 14: 641-660.

Wu, W., Bates, M., Bursian, S.J., Link, J.E., Flannery, B.M., Sugita-Konishi, Y., Wantanabe, M., Zhang, H. and Pestka, J.J., 2012a. Comparison of Emetic Potencies of the 8-Ketotrichothecenes Deoxynivalenol, 15-Acetyldeoxynivalenol, 3-Acetyldeoxynivalenol, Fusarenon X and Nivalenol. Toxicological Sciences 131: 279-291.

Wu, W., Bates, M.A., Bursian, S.J., Flannery, B., Zhou, H.R., Link, J.E., Zhang, H. and Pestka, J.J., 2013e. Peptide YY3-36 and 5-Hydroxytryptamine Mediate Emesis Induction by the Trichothecene Deoxynivalenol (Vomitoxin). Toxicological Sciences 133: 186-195.

Wu, W., Flannery, B.M., Sugita-Konishi, Y., Watanabe, M., Zhang, H. and Pestka, J.J., 2012b. Comparison of murine anorectic responses to the 8-ketotrichothecenes 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenon X and nivalenol. Food and Chemical Toxicology 50: 2056-2061.

Wu, W., Zhou, H.-R., Bursian, S.J., Link, J.E. and Pestka, J.J., 2016. Emetic responses to T-2 toxin, HT-2 toxin and emetine correspond to plasma elevations of peptide YY3–36 and 5-hydroxytryptamine. Archives of toxicology 90: 997-1007.

Wu, W., Zhou, H.-R., He, K., Pan, X., Sugita-Konishi, Y., Watanabe, M., Zhang, H. and Pestka, J.J., 2014d. Role of Cholecystokinin in Anorexia Induction Following Oral Exposure to the 8-Ketotrichothecenes Deoxynivalenol, 15-Acetyldeoxynivalenol, 3-Acetyldeoxynivalenol, Fusarenon X and Nivalenol. Toxicological Sciences 138: 278-289.

Wu, W., Zhou, H.-R., Pan, X. and Pestka, J.J., 2015. Comparison of anorectic potencies of the trichothecenes T-2 toxin, HT-2 toxin and satratoxin G to the ipecac alkaloid emetine. Toxicology reports 2: 238-251.

Xu, J., Shi, H., Ruth, M., Yu, H., Lazar, L., Zou, B., Yang, C., Wu, A. and Zhao, J., 2013. Acute toxicity of intravenously administered titanium dioxide nanoparticles in mice. PLoS One 8: e70618.

Yang, Y., Yu, S., Tan, Y., Liu, N. and Wu, A., 2017. Individual and Combined Cytotoxic Effects of Co-Occurring Deoxynivalenol Family Mycotoxins on Human Gastric Epithelial Cells. Toxins 9: 96-105.

Yoshinari, T., Sakuda, S., Furihata, K., Furusawa, H., Ohnishi, T., Sugita-Konishi, Y., Ishizaki, N. and Terajima, J., 2014. Structural Determination of a Nivalenol Glucoside and Development of an Analytical Method for the Simultaneous Determination of Nivalenol and Deoxynivalenol, and Their Glucosides, in Wheat. Journal of agricultural and food chemistry 62: 1174-1180.

Yoshizawa, T., 2013. Thirty-five years of research on deoxynivalenol, a trichothecene mycotoxin: with special reference to its discovery and co-occurrence with nivalenol in Japan. Food Safety 1: 2013002.

Zain, M.E., 2011. Impact of mycotoxins on humans and animals. Journal of Saudi Chemical Society 15: 129-144.

Zhang, J.-M. and An, J., 2007. Cytokines, inflammation and pain. International anesthesiology clinics 45: 27-37.

Zhou, H.R. and Pestka, J.J., 2015. Deoxynivalenol (Vomitoxin)-Induced Cholecystokinin and Glucagon-Like Peptide-1 Release in the STC-1 Enteroendocrine Cell Model Is Mediated by Calcium-Sensing Receptor and Transient Receptor Potential Ankyrin-1 Channel. Toxicol Sci 145: 407-417.

Zhou, T., He, J. and Gong, J., 2008. Microbial transformation of trichothecene mycotoxins. World Mycotoxin Journal 1: 23-30.

Zilfou, J.T. and Lowe, S.W., 2009. Tumor suppressive functions of p53. Cold Spring Harbor perspectives in biology 1: a001883.

Zinedine, A., Soriano, J.M., Molto, J.C. and Manes, J., 2007. Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: an oestrogenic mycotoxin. Food and Chemical Toxicology 45: 1-18.