

**DIETARY EXPOSURE TO AFLATOXIN AND FUMONISIN AMONG
CHILDREN BELOW THREE YEARS IN IRINGA, KILIMANJARO AND
TABORA REGIONS, TANZANIA**

CANDIDA PHILIP SHIRIMA

**A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY OF SOKOINE UNIVERSITY OF
AGRICULTURE. MOROGORO, TANZANIA.**

ABSTRACT

Aflatoxins and fumonisins are toxic food contaminants. Knowledge about status of human exposure to, and health effects of these toxins in Tanzania is inadequate. This study was conducted to assess the magnitude of dietary exposure to aflatoxin and fumonisin in young children by using biomarkers of exposure and assess the impact of the exposure on child growth. A total of 166 children were recruited at age of 6 to 14 months in three villages; Nyabula (Iringa region), Kigwa (Tabora region) and Kikelelwa (Kilimanjaro region) and studied at recruitment, at 6 and 12 months after recruitment. Blood and urine samples were collected and analysed for plasma aflatoxin albumin adducts (AF-alb) using ELISA and urinary fumonisin B₁ (UFB₁) using LC-MS, respectively. Anthropometric measurements were taken and growth indices, Z-scores computed. AF-alb geometric mean concentrations with 95% CI were 4.7 (3.9 - 5.6), 12.9 (9.9 - 16.7) and 23.5 (19.9 - 27.7) pg/mg albumin at recruitment, at 6 and 12 months after recruitment, respectively. At these respective sampling periods, the geometric mean UFB₁ concentrations were 313.9 (257.4 - 382.9), 167.3 (135.4 - 206.7) and 569.5 (464.5 - 698.2) pg/ml urine and the prevalence of stunted children was 44%, 55% and 56%, respectively. Poor child growth tracked over time, with 81% and 62% of children who were stunted and underweight, respectively at recruitment remaining at same status after one year. The UFB₁ concentrations at recruitment were negatively associated with length for age Z-scores (LAZ) at 6 months ($p = 0.016$) and at 12 months after recruitment ($p = 0.014$). The mean UFB₁ of the three surveys in each child was negatively associated with LAZ ($p < 0.001$) at 12 months after recruitment and length gained over the 12 months period ($p = 0.004$). There was a negative but non-significant association between AF-alb and child growth. The AF-alb and UFB₁ levels varied between survey periods and between villages. The observations suggest that the studied children were chronically exposed to aflatoxin and fumonisin and

poor child growth was prevalent and persisted over time, suggesting that exposure to aflatoxin and fumonisin may contribute to impairment of child growth.

Keywords: Aflatoxin, Biomarkers of exposure, Children, Exposure assessment, Fumonisin, Nutritional status

DECLARATION

I, Candida Philip Shirima, do hereby declare to the Senate of Sokoine University of Agriculture that this thesis is my own original work and that it has neither been submitted nor concurrently being submitted in any other institution.

Candida Philip Shirima (PhD Candidate)	Date
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The above declaration is confirmed by

Prof. Joyce L. Kinabo (Supervisor)	Date
---	-------------

Dr. Martin E. Kimanya (Supervisor)	Date
---	-------------

Dr. Yun Yun Gong (Supervisor)	Date
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LIST OF ABBREVIATIONS AND SYMBOLS

AF-alb	Aflatoxin albumin adducts
AFAR	Aflatoxin B ₁ -Aldehyde Reductase
AFB ₁	Aflatoxin B ₁
AFB ₂	Aflatoxin B ₂
AFG ₁	Aflatoxin G ₁
AFG ₂	Aflatoxin G ₂
AFM ₁	Aflatoxin M ₁
AFM ₂	Aflatoxin M ₂
AFP ₁	Aflatoxin P ₁
AFQ ₁	Aflatoxin Q ₁
CDC	Centers for Disease Control and Prevention
CI	Confidence Interval
CYP450	Cytochrome P450 enzymes
ELISA	Enzyme-Linked Immunosorbent Assay
FB ₁	Fumonisin B ₁
FB ₂	Fumonisin B ₂
FB ₃	Fumonisin B ₃
GEMS	Global Environment Monitoring System
GST	Glutathione S-Transferases
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HIV	Human Immunodeficiency Virus
IARC	International Agency for Research on Cancer
LAZ	Length for Age Z-score

LC-MS	Liquid Chromatography-Mass Spectrometry
LOD	Limit of Detection
mEH	microsomal Epoxide Hydrolase
MOHSW	Ministry of Health and Social Welfare
NBS	National Bureau of Statistics
NTD	Neural Tube Defects
OR	Odds Ratio
PMTDI	Provisional Maximum Tolerable Daily Intake
SES	Socio-economic Status
SUA	Sokoine University of Agriculture
TFDA	Tanzania Food and Drugs Authority
UFB ₁	Urinary Fumonisin B ₁
UNICEF	United Nations Children's Fund
URT	United Republic of Tanzania
WAZ	Weight for Age Z-score
WHO	World Health Organisation
WLZ	Weight for Length Z-score

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Aflatoxins and fumonisins are among the main types of mycotoxins of significant public health concern due to their widespread contamination in foodstuffs as well as chronic and acute health effects in human (IARC, 2002; Bakirdere *et al.*, 2012; Wu, 2014). Aflatoxins are toxic secondary metabolites produced by fungi of *Aspergillus* species. Aflatoxin contamination is prominent in vital food commodities such as maize, groundnuts, tree nuts and cotton seed (IARC, 1993; O'Riordan and Wilkinson, 2008; Bhat *et al.*, 2010; Milićević *et al.*, 2010). Fumonisin are another type of mycotoxins, produced by *Fusarium* fungi and largely contaminate maize (IARC, 2002; Bulder *et al.*, 2012). Consumption of mycotoxin contaminated food is the major source of human exposure to these health risk toxins (Pitt *et al.*, 2012; Shephard *et al.*, 2013a; Torres *et al.*, 2014a). It is estimated that up to 25% of world's cereal crops is contaminated with mycotoxins (Bhat *et al.*, 2010). Additionally, exposure to mycotoxins or their metabolites can occur through consumption of contaminated animal products such as milk, meat and eggs, which results from consumption of contaminated feeds by animals (Tchana *et al.*, 2010; Pitt *et al.*, 2012; Zheng *et al.*, 2013).

Mycotoxins have diverse chemical, biological and toxicological properties. These properties make their toxic effects to vary and also depend on toxin intake, duration of exposure, animal species, age, physiological status, and eventual synergism among mycotoxins simultaneously present in food or feed (Piva and Galvano, 2007). Acute and chronic exposure to aflatoxins and fumonisins have been implicated in various health effects both in human and animals (IARC, 2002; Sherif *et al.*, 2009; Wu *et al.*, 2014).

Aflatoxin B₁, one of the most potent forms of the aflatoxins is a human carcinogen (IARC, 1993) as well as being associated with suppressed immunity (Turner *et al.*, 2003), child growth impairment (Gong *et al.*, 2002, 2004; Shuaib *et al.*, 2010; Shouman *et al.*, 2012), low birth weight (Shuaib *et al.*, 2010), hepatomegaly (Gong *et al.*, 2012), liver failure and fatalities due to acute poisoning (Probst *et al.*, 2007). Fumonisin contamination in maize has been linked with oesophageal cancer in humans (Rheeder *et al.*, 1992; Yoshizawa *et al.*, 1994), neural tube defect in humans (Marasas *et al.*, 2004; Missmer *et al.*, 2006), growth faltering in infants (Kimanya *et al.*, 2010) and retarded growth in experimental animals (Dilkin *et al.*, 2003). Fumonisin B₁, the most common analogue fumonisin has been classified by the International Agency for Research on Cancer (IARC) as “possibly carcinogenic in human” (IARC, 2002). Basically, there is sufficient evidence from animal models and human epidemiological data to conclude that mycotoxins have serious consequences in health.

Mycotoxins are prevalent contaminants of cereals and nuts in tropical and sub-tropical areas of the world where temperature and humidity conditions favour fungal growth and production of the toxins (Bhat *et al.*, 2010). Mycotoxins contamination can occur in food at any point along the value chain starting from farm before a crop is harvested, during harvest or post harvest operations such as handling, transportation, storage and processing (Schatzmayr and Streit, 2013). Aflatoxins and fumonisins have been estimated to be widespread in major dietary staples in Africa (Wagacha and Muthomi, 2008; Wild and Gong, 2010; Bulder *et al.*, 2012; Gnonlonfin *et al.*, 2013; Marin *et al.*, 2013; Schatzmayr and Streit, 2013).

Maize (*Zea mays* L.) is the major food product that is highly susceptible to both aflatoxin and fumonisin contamination (Shephard, 2014). Maize is the dominant staple food crop and

also a cash crop grown in all agro-ecological zones in Tanzania (URT, 2010; Ranum *et al.*, 2014). Maize constitutes 31% of the total food production and more than 75% of cereal consumption in Tanzania as well as the most common cereal, consumed at 5.8 days per week (URT, 2010). The estimated maize consumption in Tanzania is 128 g/person/day (Ranum *et al.*, 2014). Consequently, maize forms the basic ingredient in complementary foods for children consumed mainly as thin porridge (*uji*) as well as stiff porridge (*ugali*). The households in rural areas consume more maize compared to urban households (Smith and Subandoro, 2007). Despite the potential of maize as major staple food, the crop is highly susceptible to contamination with aflatoxins and fumonisins. Groundnut is also one of the common ingredients in food, used in composite flour for children porridge and also added in sauce dishes or eaten as a snack. However, the crop is highly prone to aflatoxins contamination (Pitt *et al.*, 2012; Torres *et al.*, 2014b; Andrade and Caldas, 2015). In Tanzania, groundnuts is one of the crops cultivated most frequently (20.6%) after maize (90.5%), kidney beans (36.5%), cassava (29.3%) and rice (23.0%) (URT, 2010).

1.2 Problem Statement

Studies conducted in Iringa, Kilimanjaro, Manyara, Mbeya, Morogoro, Mtwara, Rukwa, Ruvuma, Shinyanga and Tabora regions of Tanzania revealed contamination of maize with either aflatoxins or fumonisins or co-occurrence of both toxins (Kimanya *et al.*, 2007, 2008a, b; Manjula *et al.*, 2009; Mboya *et al.*, 2011; Abt Associates Inc., 2013; Kamala *et al.*, 2015). One of the studies (Kimanya *et al.*, 2008a) reported that about 18% of the samples of home grown maize were contaminated with aflatoxin at levels up to 158 µg/kg, with 12% of these contaminated with total aflatoxin levels above the Tanzanian limit of 10 µg/kg. In this study, fumonisins have been detected in 52% of home grown maize samples at concentrations of up to 11 048 µg/kg, with 15% of these positive samples exceeding 1000 µg/kg, the maximum tolerable limit for fumonisins in maize (Kimanya *et al.*, 2008b).

That study also determined that both aflatoxins and fumonisins co-occurred in 10% of the maize samples tested and a more recent study (Kamala *et al.*, 2015) has determined co-occurrence of aflatoxins and fumonisins in 45% of maize samples. Another Tanzanian study found unacceptable levels of aflatoxin contamination in groundnuts, whereby 19% of tested samples from Manyara, Mtwara and Shinyanga regions were detected with total aflatoxin at levels exceeding the maximum limit of 10 µg/kg (Abt Associates Inc., 2013). Apart from maize and groundnuts, other studies in Tanzania have detected aflatoxins and fumonisins in cassava (Manjula *et al.*, 2009), aflatoxins in cured fish (Mugula and Lyimo, 1992) and in commercial locally processed cereal based complementary foods (Rushunju *et al.*, 2013), aflatoxin M₁ (aflatoxin B₁ metabolite) in cow's milk (Njapau *et al.*, 2006) and recently, breast milk has been found to be contaminated with aflatoxin M₁ and fumonisin B₁ (Magoha *et al.*, 2014a, b). Considering occurrence of aflatoxins and fumonisins in the potential food crops, it is very likely that consumers are exposed to a mixture rather than a single mycotoxin and therefore may be predisposed to health effects in an additive or interactive (synergistic) manner (Grenier and Oswald, 2011; De Ruyck *et al.*, 2015). Combined effects of co-exposure to aflatoxin and fumonisin have been reported in experimental animals. Such effects include erythrocyte membrane damage and hemoglobin changes due to sub-chronic treatment with aflatoxin B₁ and fumonisin in rats (Abdel-Wahhab *et al.*, 2014). Other synergistic effects are decreased deposition of abdominal fat and reduced plasma levels of triglycerides and very low-density lipoproteins in chicken (Siloto *et al.*, 2013). The prevailing status of mycotoxins contamination in food presents a unique challenge to food safety considering that, agriculture in rural communities of Tanzania is dominated by smallholder subsistence farming system (URT, 2010) whereby maize and other food crops are produced and consumed within the households and communities without prior inspection or control of their safety.

The major source of human exposure to mycotoxins is through consumption of contaminated food (Pitt *et al.*, 2012). Other sources of exposure are inhalation of mycotoxins occurring in dust, air and working environment (Straumfors *et al.*, 2014; Fromme *et al.*, 2015) as well as dermal contact surfaces (Boonen *et al.*, 2012). Since aflatoxins and fumonisins are prevalent contaminants in maize and groundnuts, which comprise basic ingredients of complementary foods, children consuming such foods without access to uncontaminated food are at risk of toxicity due to these contaminants. In addition to exposure from consuming contaminated complementary foods, children exposure to mycotoxins may also occur *in utero* and through breast milk (Khlanguiset *et al.*, 2011), therefore predisposing children to the risk of chronic exposure from a very early stage of life. Under circumstances of exposure to toxic chemicals, health and growth of children are likely to be affected more than that of adults, because they are distinctively vulnerable due to having less developed detoxification capability, rapid rate of cell division and growth and greater exposure relative to body size (Makri *et al.*, 2004; Erkekoğlu *et al.*, 2008; Raiola *et al.*, 2015). The exposure may exacerbate susceptibility to malnutrition and other health effects since the toxins interfere with growth and development processes in the body (Gong *et al.*, 2002, 2008a; Makri *et al.*, 2004; Erkekoğlu *et al.*, 2008; Kimanya *et al.*, 2010).

Good nutritional status from conception to the age of two years is particularly important since this is a critical period of life because all organs are being formed and malnutrition at this age may cause irreversible damage to physical growth and brain development (Black *et al.*, 2008). Poor childhood growth is prevalent in Tanzania, where the proportions of stunting, underweight and wasting in children under five years old are 42%, 16% and 5%, respectively (NBS and ICF Macro, 2011). Impaired child growth is an indicator of poor nutrition and infection (Black *et al.*, 2013) and is associated with increased susceptibility to

disease and mortality, impaired cognitive development and reduced educational achievement as well as reduced work capacity and productivity during adulthood (Victora *et al.*, 2008; URT/UNICEF, 2010; Black *et al.*, 2013). In Tanzania, there has been a high level of interventions to improve child nutritional status such as vitamin A supplementation, food fortification, immunisation against vaccine preventable diseases and nutrition education. As a result of such initiatives, there has been at national level a decreased trend of prevalence of stunting for children under five years from 48% in 1999 to 42% in 2010 and underweight from 25% to 16% during the same period (NBS and ICF Macro, 2011). Nevertheless, despite the reduced trend, the prevailing rate of impaired growth is still unacceptably high (URT/UNICEF, 2010). Likewise, a substantial number of children still suffer from other forms of under-nutrition including low birth weight and micronutrients deficiency (URT/MOHSW, 2011). This suggests that other factors in addition to nutrition and infection, which may have an adverse impact on childhood growth have not been accurately determined and therefore hinder success of nutritional intervention programmes. To date, any effect that mycotoxins such as aflatoxins and fumonisins may have in relation to child growth and development has not been fully investigated in Tanzania. Since these natural contaminants are prevalent in food crops, mainly cereals and groundnuts that form basic ingredients of complementary foods, children consuming such foods are exposed to the contaminants and the subsequent health effects.

1.3 Justification

Despite the prevailing contamination of aflatoxins and fumonisins in complementary food ingredients (as already established from food analyses), there is inadequate knowledge about magnitude of dietary exposure of young children in Tanzania to these potent fungal toxins. This gap is associated with lack of studies that have estimated aflatoxins exposure

and inadequacy in the methods that have previously been used to estimate fumonisin exposure. These shortfalls substantiate the rationale to investigate the magnitude of exposure of children to these toxins by using a reliable method. The method for estimating exposure to mycotoxins in this study is the use of exposure biomarkers that measures the concentrations present in biological matrices such as blood and urine (Turner *et al.*, 1999; Shephard *et al.*, 2007; Milićević *et al.*, 2010; Kensler *et al.*, 2011; Routledge and Gong, 2011) and provide direct evidence of actual human exposure (Sexton *et al.*, 2004). Biomarker assessment methods provide useful indication of dietary intake of mycotoxins and are the most accurate and reliable methods in assessing exposure at individual level when compared to assessment of dietary exposure by using food based methods. Furthermore, biomarker method takes into consideration variations in food intake and is critical for assessment of possible human health outcomes due to mycotoxins ingestion and setting of limits for mycotoxins control (Cano-Sancho *et al.*, 2010; Milićević *et al.*, 2010; Baldwin *et al.*, 2011). In view of prevailing contamination of aflatoxins and fumonisins in complementary food ingredients coupled with prevailing high prevalence of impaired childhood growth in Tanzania, there is also rationale to investigate whether typical levels of the mycotoxin exposure could be associated with child growth impairment in the country. Studies to evaluate dietary exposure to mycotoxins by using biomarkers of exposure have not been conducted in any of the Tanzanian human population including infants and children. Consequently, there has not been any study to link child growth performance and magnitude of mycotoxin exposure from biomarkers assessment.

Furthermore, it is also important to note that, most of the records on child malnutrition in Tanzania are from cross-sectional studies, which have their limitations. Longitudinal studies are fundamental in assessing nutritional status and tracking of growth determinants within a reasonable length of study period. It was important therefore to conduct a longitudinal study to assess the prevalence of child malnutrition and determine factors

associated with malnutrition especially in rural areas where this risk is particularly high, so as to facilitate actions for addressing the problem at the community level.

It is anticipated that data generated from this study will form a useful evidence for informing about magnitude and severity of dietary exposure of young children to aflatoxin and fumonisin and consequent association with growth performance. This information is important for developing food safety and nutrition measures to prevent children and the general public from exposure to the harmful mycotoxins and the consequent health risks.

1.4 Objectives

1.4.1 Overall objective

To assess the magnitude of dietary exposure to aflatoxin and fumonisin and its impact on growth in young children.

1.4.2 Specific objectives

The specific objectives were:-

- (i) To determine dietary exposure of children to aflatoxin by using blood biomarker.
- (ii) To determine dietary exposure of children to fumonisin by using urine biomarker.
- (iii) To assess children's nutritional status.
- (iv) To examine the association of child growth and exposure to aflatoxin and fumonisin.

1.5 Research Questions

The following research questions were formulated to guide the study in order to achieve the overall objective:-

- (i) Are young children in Tanzania exposed to aflatoxin through dietary intake of contaminated food? What proportion of children population is exposed and what are the determinant factors? Is there a difference in levels of exposure to aflatoxin between different periods of the year, age, child feeding practices and geographical locations?
- (ii) Are young children in Tanzania exposed to fumonisin through dietary intake of contaminated food? What proportion of children population is exposed and what are the determinant factors? Is there a difference in levels of exposure to fumonisin between different periods of the year, age, child feeding practices and geographical locations?
- (iii) What is the prevalence of stunting, underweight and wasting in children? What are the factors contributing to poor child growth?
- (iv) Is there any association between poor child growth and exposure to aflatoxin and fumonisin?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Overview of Aflatoxins

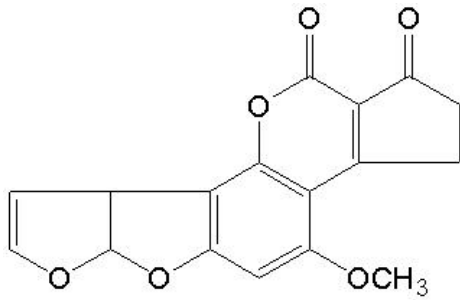
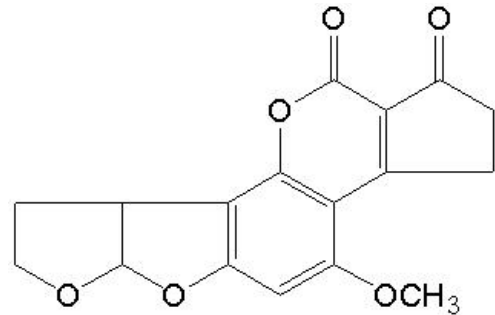
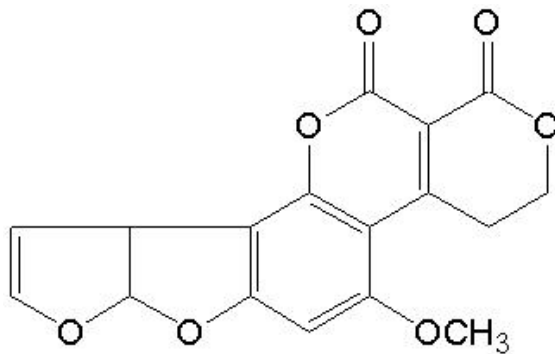
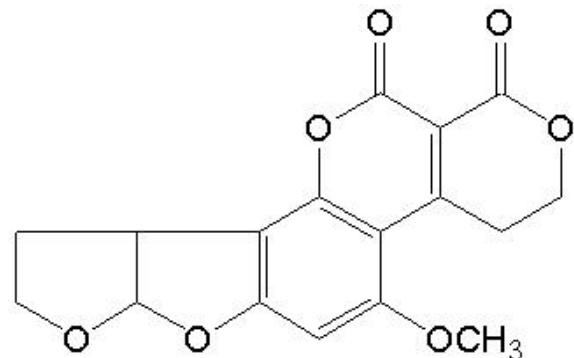
Aflatoxins are highly toxic secondary metabolites produced mainly by fungi of *Aspergillus* spp mainly *A. flavus* and *A. parasiticus* (IARC, 1993; Bhat *et al.*, 2010) and rarely by *A. nomius* (Olsen *et al.*, 2008). The *Apergillus* species are prevalent in tropical and sub-tropical areas in the world where warm temperature and humid conditions favour fungal growth and subsequent production of aflatoxins (IARC, 1993). Aflatoxins were first identified in 1961 as a result of the outbreak of the unknown disease which caused mortality in turkeys and other animals in the England (Sargeant *et al.*, 1961). The unknown disease was named Turkey “X” and was attributed to heavy infestation of *A. flavus* in the suspected animal feed. Subsequently, feed samples were analysed and some compounds were detected, which afterwards were named aflatoxins (Lancaster *et al.*, 1961). The term aflatoxin was built up by combining a letter “a” representing *Apergillus* genus, “fla” for *flavus* species and “toxin” for poison (Ellis *et al.*, 1991).

There are various types of naturally occurring aflatoxins, which have been identified (IARC 1993; 2002). However, aflatoxins of major importance are aflatoxin B₁ (AFB₁), aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁) and aflatoxin G₂ (AFG₂). AFB₁ and AFB₂ are produced by fungi of *A. flavus* while *A. parasiticus* produces AFB₁, AFB₂, AFG₁ and AFG₂ (Kurtzman *et al.*, 1987, IARC, 1993, Huchchannavar and Balol, 2011). The letters B and G refers to blue and green fluorescence colours, respectively, produced by the toxins under ultraviolet light on thin-layer chromatography while number 1 and 2 represents different homologues (Huchchannavar and Balol, 2011; Quadri *et al.*, 2013).

Out of the four major types of aflatoxins, AFB₁ is the most potent toxin as well as carcinogenic (IARC, 1993, 2002), which has received big attention in epidemiological researches. In respect to the magnitude of aflatoxin potency, both for the acute and chronic toxicity, the order of magnitude is AFB₁ > AFG₁ > AFB₂ > AFG₂ (IARC, 1993). Likewise, in terms of magnitude of occurrence, AFB₁ is most frequent accounting for about 70% of the total aflatoxin contents in foods although this may also vary, followed by AFG₁, while AFB₂ and AFG₂ occur at much lower levels (Horn, 2003; Yu *et al.*, 2014). Aflatoxin B₁ and B₂ can undergo hydroxylation to form aflatoxin M₁ (AFM₁) and aflatoxin M₂ (AFM₂) metabolites, respectively, which can be excreted in milk of lactating mammals due to consumption of food or feed, which has been contaminated with aflatoxins (Quadri *et al.*, 2013). Aflatoxin M₁ has been classified by the IARC as “possibly carcinogenic to humans” (Group 2B) (IARC, 2002).

2.1.1 Chemistry of aflatoxins

Purified aflatoxins are colourless to pale-yellow crystalline substances (Pitt *et al.*, 2012). They are strongly fluorescent in ultraviolet light with AFB₁ and AFB₂ emitting blue fluorescence, while AFG₁ and AFG₂ emit green fluorescence (Huchchannanavar and Balol, 2011; Pitt *et al.*, 2012; Quadri *et al.*, 2013). Aflatoxins are slightly soluble in water (Nabok *et al.*, 2011), insoluble in non-polar solvents, freely soluble in polar organic solvents (such as methanol, chloroform and dimethyl sulfoxide) and very stable up to temperatures above 100 °C (Quadri *et al.*, 2013). Aflatoxins are degraded by reaction with ammonia or sodium hypochlorite (Kanungo and Bhand, 2013; Abu-El-Zahab *et al.*, 2013). The toxins are unstable to ultraviolet light in the presence of oxygen, to pH of less than 3 and above 10, and also to oxidizing agents (Quadri *et al.*, 2013). In food, aflatoxins are not readily degraded under the normal food cooking conditions (Bullerman and Bianchini, 2014). The chemical structures of aflatoxins are presented in Fig.1.

Aflatoxin B₁ (C₁₇ H₁₂ O₆)Aflatoxin B₂ (C₁₇ H₁₄ O₆)Aflatoxin G₁ (C₁₇ H₁₂ O₇)Aflatoxin G₂ (C₁₇ H₁₄ O₇)

Source: Zain, (2011).

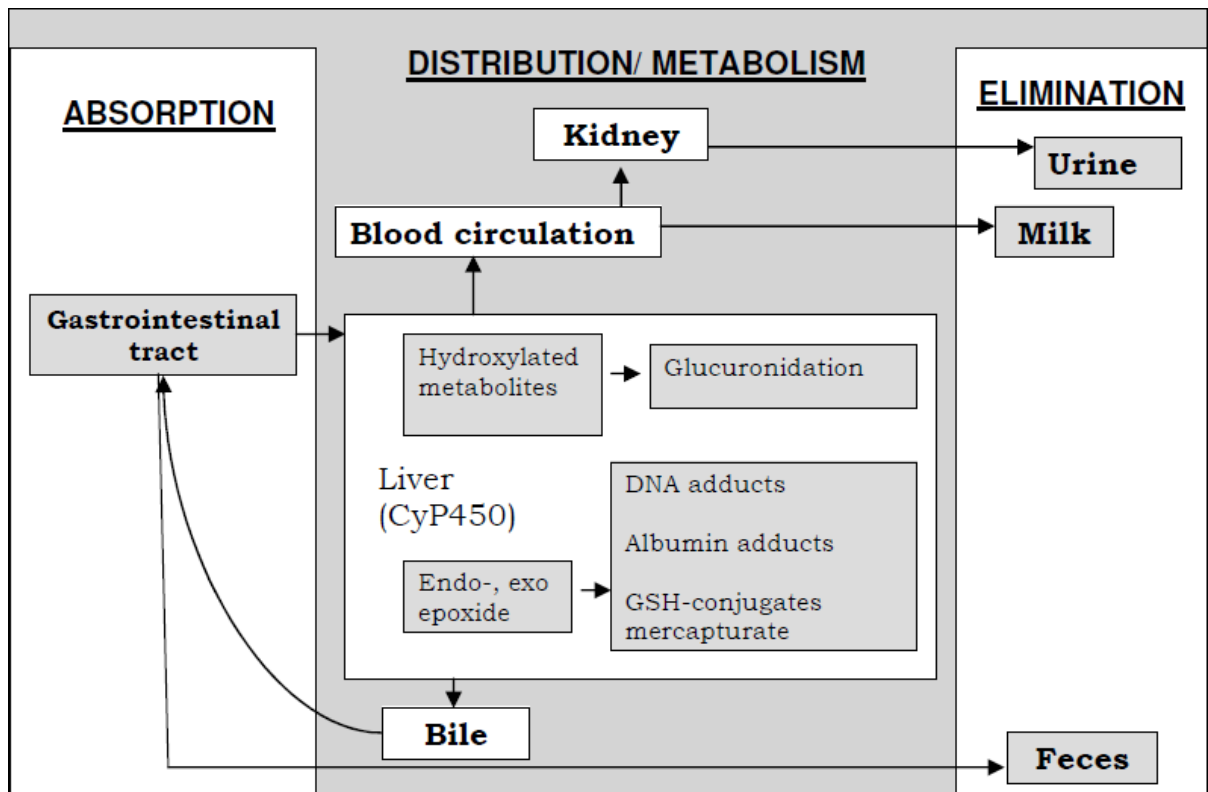
Figure 1: The chemical structure of aflatoxins

2.1.2 Absorption, distribution, metabolism and excretion of aflatoxins

The oral intake of aflatoxin contaminated foods is the main route of aflatoxin exposure. Also by route of food ingestion, aflatoxin exposure can occur through ingestion of aflatoxins carried over from feed into animal products such as milk, meat and their products (Bhat *et al.*, 2010; Völkel *et al.*, 2011). In addition, breastfed children may be exposed to AFM₁ from breast milk if lactating mothers have consumed aflatoxin contaminated food (Khlanguiset *et al.*, 2011; Magoha *et al.*, 2014a; Diaz and Sánchez, 2015). Other important routes of exposure are through inhalation of aflatoxin contaminated

dust as a result of occupational exposure, through handling and processing of contaminated crops, particularly, during undertakings such as shelling and processing (Bbosa *et al.*, 2013a) and direct dermal contact (Zain, 2011). Animal studies found that in normal conditions, 50% of ingested AFB₁ is quickly absorbed in the duodenum and reach the liver by the portal system (Kumagai, 1989; Coulombe *et al.*, 1991).

Aflatoxin B₁ is highly lipo-soluble and after ingestion, it is efficiently absorbed across the cell membrane from the duodenal region of the small intestine (Kumagai, 1989) and from respiratory tract in the case of occupational airway exposure (Larsson and Tjälve, 2000) into the blood stream. Aflatoxin B₁ is concentrated in the liver and in lesser amounts in the kidneys (Wogan *et al.*, 1967). Liver is a target site for both acute and chronic toxicity due to aflatoxin exposure. In liver, AFB₁ is activated by a number of cytochrome P450 enzyme (CYP450) family and bio transformed to several metabolic products before excretion. One of the major AFB₁ metabolites is AFB₁-8,9-epoxide, a highly active molecule which binds covalently to deoxyribonucleic acid (DNA) and produce AFB₁-N7-guanine (Raney *et al.*, 1993), as well as other cellular macromolecules. In addition to DNA, the epoxide can also bind to the serum albumin to form aflatoxin albumin (AF-alb) or lysine adducts, which subsequently enter the systemic circulation (Essigmann *et al.*, 1982; Forrester *et al.*, 1990; Sabbioni and Wild, 1991). The summarised mechanism of aflatoxin absorption, biotransformation and excretion is illustrated in Fig. 2.

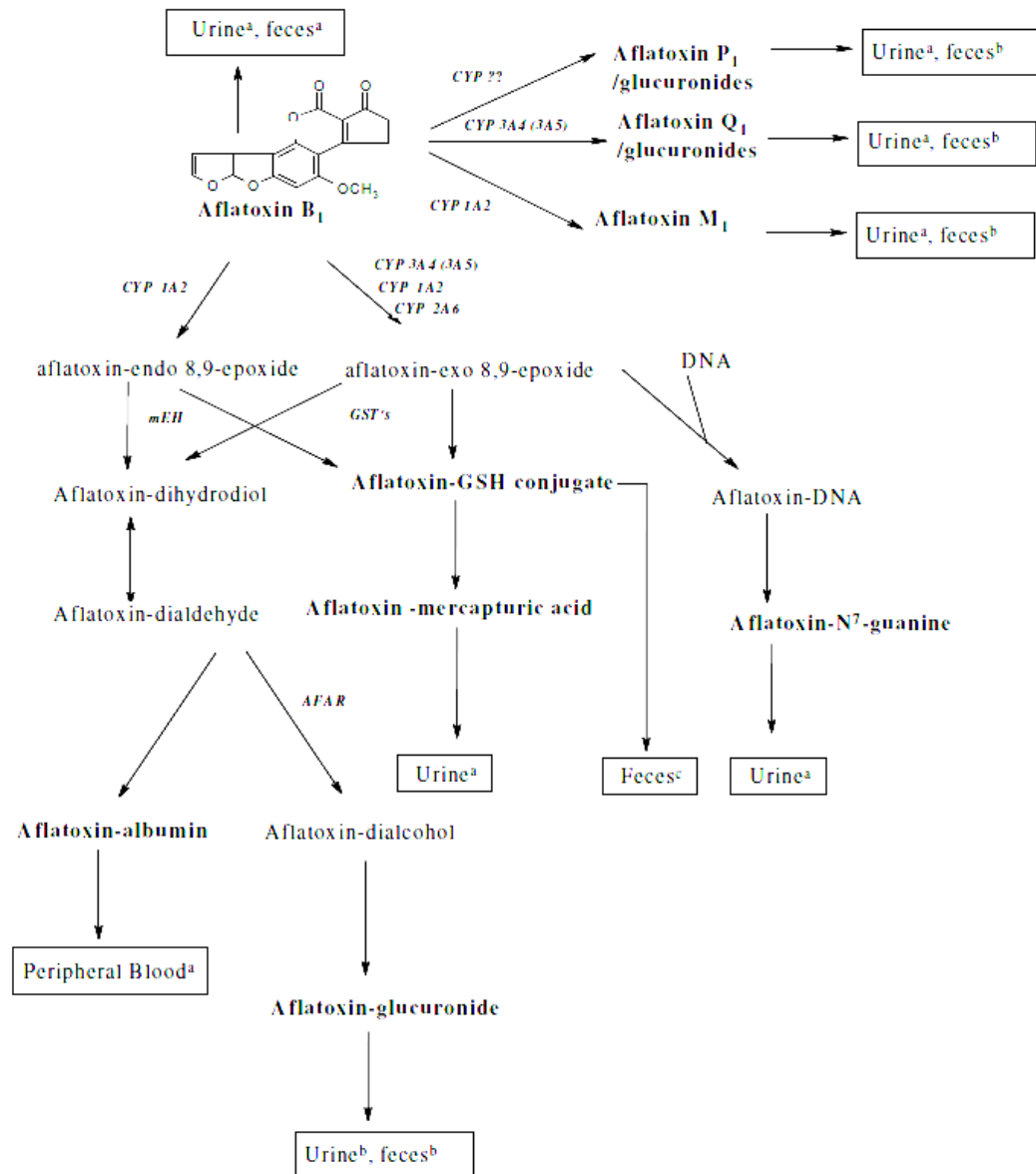


Source: Polychronaki, (2007a).

Figure 2: Absorption, metabolism and elimination of aflatoxins

The mechanism of formation of AFB₁-8,9-epoxide and subsequent binding to DNA and proteins to form adducts is believed to be accountable for the biological effects and damage of the cellular targets (i.e DNA and proteins) (Wild and Turner, 2002a; Bbosa *et al.*, 2013b). This mechanism causes dysfunction of normal process of the cell (Wild and Hall, 2000a), therefore results into reduced mitochondrial function and causes damage to critical cellular components such as DNA, lipids and proteins (Essigmann *et al.*, 1982; Wild and Turner, 2002a). The formation of bonds between AFB₁ and DNA alters the structure and biological activity of DNA, hence leading to the mutagenic and carcinogenic mechanism of the AFB₁ (Essigmann *et al.*, 1982; Hsieh and Atkinson, 1991). Apart from the formation of AFB₁-8,9-epoxide, AFB₁ in liver is also metabolised to form hydroxylated metabolites called aflatoxin M₁ (AFM₁), aflatoxin Q₁ (AFQ₁), aflatoxin P₁ (AFP₁) and aflatoxicol (Wild and Turner, 2002a; Egner *et al.*, 2003; Kensler *et al.*, 2011). The AFM₁ metabolite

can further be activated to form a new metabolite called AFM₁-8,9-epoxide, which binds to DNA to form AFM₁-N7-guanine (Egner *et al.*, 2003; Verma, 2004). AFB₁ metabolites in liver can also be excreted into the small intestine via bile (Bbosa *et al.*, 2013b) and can get reabsorbed and returned to the liver via portal circulation (enterohepatic circulation). Both absorbed AFB₁ and its metabolites are excreted in urine, faeces and milk. Unabsorbed aflatoxin from gastro-intestinal tract and absorbed AFB₁ metabolites from biliary excretion to the intestine are both excreted via faeces (Hsieh *et al.*, 1993). The urinary route forms an important pathway of excretion of absorbed aflatoxin and aflatoxin metabolites (Groopman *et al.*, 1985; Egner *et al.*, 2003). Breast feeding mothers who consume aflatoxin contaminated foodstuffs may excrete aflatoxin metabolites in their milk (IARC, 2002; Polychronaki *et al.*, 2007b). Details of aflatoxin biotransformation pathways are illustrated in Fig. 3.



^aEvidence of excretion of metabolite from experimental and human studies

^bScarce or no evidence available

^cNo human data, only experimental evidence available

CYP = cytochrome P450 enzyme family

GST = glutathione S-transferases

mEH = microsomal epoxide hydrolase

AFAR= aflatoxin B₁-aldehyde reductase

Source: Mykkänen *et al.* (2005).

Figure 3: Pathways of aflatoxin B₁ biotransformation and excretion

2.2 Impact of Aflatoxins on Human Health

Poisoning that result from the ingestion of aflatoxins is called aflatoxicosis (Çelik *et al.*, 2005). There are two forms of aflatoxicosis which have been identified; the first is acute severe intoxication, which results in direct liver damage and subsequent illness or death. The second form is called chronic sub-symptomatic exposure. Dose and duration of exposure to aflatoxins have clear major effects on the toxicology and may cause a range of health consequences. Chronic sublethal doses have nutritional and immunologic consequences, while all doses have a cumulative effect on the risk of cancer (Williams *et al.*, 2004).

2.2.1 Liver carcinogen

Aflatoxins have been studied extensively and cause detrimental health effects in body system and organs of both humans and animals (Bbosa *et al.*, 2013a). Aflatoxins are potent hepatocarcinogens in animals and human epidemiological studies have provided sufficient evidence of carcinogenicity, which has led to AFB₁ being classified as “group 1 human carcinogen” (IARC, 2002). High incidence of hepatocellular carcinoma (HCC) occurs most commonly in populations where chronic infection with hepatitis B virus (HBV) and chronic dietary exposure to aflatoxins are also prevalent. Synergistic interaction between AFB₁ and HBV in causing liver cancer has been demonstrated in Chinese population (IARC, 1993; Groopman *et al.*, 2005; Wild and Montesano, 2009; Kensler *et al.*, 2011). Cancer effects due to aflatoxin is attributed to its ability to form adducts through the binding of DNA with AFB₁-8,9-epoxide metabolite, that is generated during AFB₁ biotransformation. Formation of adducts causes alteration of DNA, a gene mutation, which is believed to be responsible for liver cancer (IARC, 1993; Wild and Turner, 2002a; Bbosa *et al.*, 2013a). From a public health view point, these observations demonstrate that

measures to reduce aflatoxin exposures along with HBV vaccination could have a potential impact on minimising the burden of liver cancer (Kensler *et al.*, 2003).

2.2.2 Immune suppression

Following ingestion of aflatoxins through contaminated food, the intestine is the primary target for toxin induced damage (Turner *et al.*, 2012). Effects on the gastro-intestinal tract as a result of aflatoxin exposure have been reported in several animal studies (IARC, 1993; Applegate *et al.*, 2009). Intestine impairment disrupts absorption of nutrients in the body, which contributes to nutritional deficiencies. The immunomodulatory effects of aflatoxins have been considered in experimental studies in cell models and animals studies (IARC, 2002; Williams *et al.*, 2004). In Gambian children, higher exposure to aflatoxin was associated with lower salivary immunoglobulin A (IgA) (Turner *et al.*, 2003). In Ghana, a study reported that human immunodeficiency virus (HIV) positive individuals with higher levels of aflatoxin biomarker, had significantly lower percentages of CD4+ T regulatory cells compared with HIV positive individuals with lower AFB₁ biomarker levels (Jiang *et al.*, 2008). Although the available findings do not permit conclusion to be drawn about impact of aflatoxin exposure on human immunity, the data still suggests that immune parameters could be affected in populations exposed chronically to aflatoxins (Pitt *et al.*, 2012). Impaired immune function contributes to increased potential to susceptibility to infectious diseases in exposed population and particularly the vulnerable groups.

2.2.3 Impairment of childhood growth

Studies in animal species indicate that chronic exposure to aflatoxin can severely affect growth and development (Pitt *et al.*, 2012). In the veterinary field, exposure in animals has been linked with reduced weight gain, reduced feed intake and uptake of nutrients,

impaired feed conversion efficiency, impaired immunity and growth faltering (Han *et al.*, 2008; Andretta *et al.*, 2012; Da Rocha *et al.*, 2014; Rezaei *et al.*, 2014).

In human studies, aflatoxin exposure *in utero*, during breast feeding and complementary feeding has been implicated with child growth impairment. One of the earliest studies reported the linkage between aflatoxin exposure and kwashiorkor in Sudanese children (Hendrickse *et al.*, 1982). High prevalence of wasting (a reflection of acute malnutrition) was associated with consumption of cereals contaminated with high levels of aflatoxin in Kenya (Okoth and Ohingo, 2005). A cross-sectional study in Togo and Benin found an inverse association between AF-alb concentration and growth indicator Z-scores in children (Gong *et al.*, 2002). In Benin, a longitudinal study demonstrated strong negative correlation between levels of AF-alb and height increase over eight months of follow-up, with children in the highest exposure group being associated with mean reduction of 1.7 cm in height compared with the lowest exposure group (Gong *et al.*, 2004). Association has also been found between exposure to aflatoxin *in utero* and growth impairment during the first year of life in Gambian children (Turner *et al.*, 2007). A study in Ghana reported that mothers detected with higher levels of AFB₁-lysine during pregnancy were more likely to have low birth weight babies compared to the mothers who were found with lower levels (Shuaib *et al.*, 2010).

However, despite the associations reported on aflatoxin exposure and growth impairment, the biological mechanisms of action by which aflatoxin may exert an effect on growth are currently not clear and therefore merits further investigation (Pitt *et al.*, 2012). Possible hypotheses include compromised intestinal integrity and absorption of nutrients through altered barrier function due to endothelial cell toxicity or immune suppression (Gong *et al.*, 2008a; Smith *et al.*, 2012). Other possible reasons include inhibition of protein synthesis

due to binding of AFB₁ with DNA, ribonucleic acid (RNA) and proteins and therefore cause interference with enzymes and substrates that are required for processes involved in protein synthesis (Bbosa *et al.*, 2013a). However, further investigation of the impact of aflatoxin on health is needed due to the fact that health risks from aflatoxins may be complicated by exposure to other types of mycotoxins which co-occur in the same food (Strosnider *et al.*, 2006) as well as other confounding factors.

2.2.4 Acute toxicity and death

Acute exposure to aflatoxins can result in aflatoxicosis, which manifests as severe, acute hepatotoxicity. Early symptoms of severe aflatoxicosis can include anorexia, malaise, and low-grade fever. Acute high-level exposure can progress to vomiting, abdominal pain, jaundice, hepatic failure and death (Strosnider *et al.*, 2006). Acute dietary exposure to high aflatoxin levels has been implicated in recurring outbreaks of acute toxicity and deaths. Between the years 2004 and 2005, consecutive outbreaks of acute aflatoxicosis caused by aflatoxin contamination in home grown maize occurred in Kenya and caused 125 confirmed deaths out of 317 cases of acute liver failure (Gieseke and CDC, 2004; Probst *et al.*, 2007), thus emphasising the serious public health problem due to aflatoxin exposure.

2.3 Overview of Fumonisin

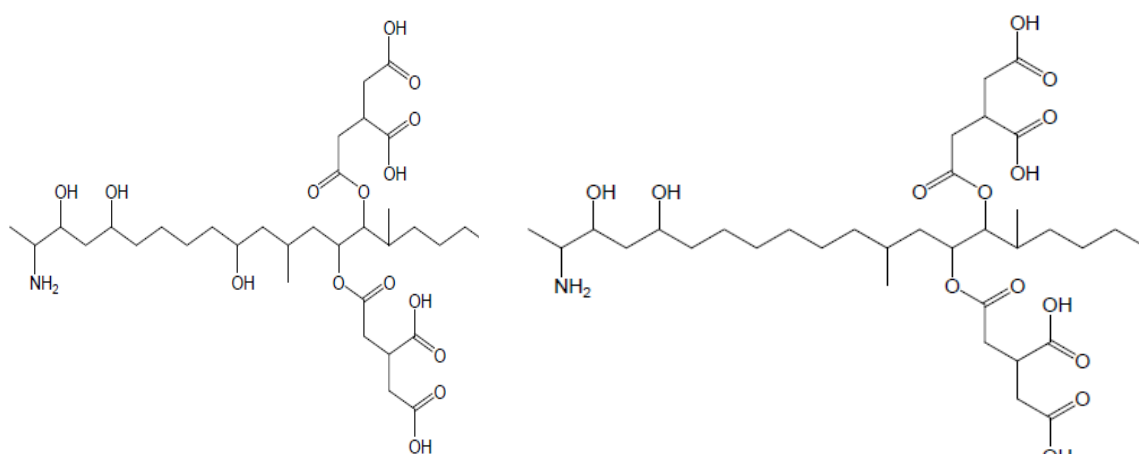
Fumonisin are a group of mycotoxins produced by *Fusarium* species, primarily *F. verticillioides* and *F. proliferatum*. Fumonisin B₁ (FB₁), fumonisin B₂ (FB₂) and fumonisin B₃ (FB₃) are the main naturally occurring fumonisin analogues (Shephard *et al.*, 1996; IARC, 2002; Rheeder *et al.*, 2002; Bulder *et al.*, 2012). Fumonisin B₁ is the most common and toxic, most thoroughly studied and most abundant of the fumonisin family, which accounts for about 70% of the total fumonisin contamination in food and feed (Shephard *et al.*, 1996; Marasas, 2001; Yu *et al.*, 2014). Fumonisin were first isolated from cultures of

F. verticillioides strains in 1988 in South Africa and their chemical structures were elucidated (Gelderblom *et al.*, 1988). The discovery of fumonisins was a result of the search for a possible cause of the high incidence of esophageal cancer in the Transkei region of South Africa (Marasas, 2001). Worldwide, fumonisins predominantly contaminate maize and maize products. Limited data has been produced for natural occurrence of fumonisins on other cereal produce apart from maize before 1996 (Shephard *et al.*, 1996). However, in later years, fumonisins were detected in other food products. For example, fumonisin B₁ has also been detected in sorghum and sorghum beer (Roger, 2011; Ratnavathi *et al.*, 2012), wheat (Stanković *et al.*, 2012; Cendoya *et al.*, 2014; Li *et al.*, 2015), rice (Bansal *et al.*, 2011) and fumonisin FB₂ in coffee beans, wine and beer (Scott, 2012). This adds to the list of foodstuffs and feedstuffs other than maize in which fumonisins have been found. Based on their ubiquitous natural occurrence in these popular food products, the toxins have elicited considerable food safety attention (Stoev, 2013; Darwish *et al.*, 2014). Compared to aflatoxins, fumonisins are less documented as they were discovered about 30 years after aflatoxins. This information gap is a matter of concern especially in countries where people consume contaminated maize based foods without being aware of danger associated with these harmful toxins (Fandohan *et al.*, 2004).

2.3.1 Chemistry of fumonisins

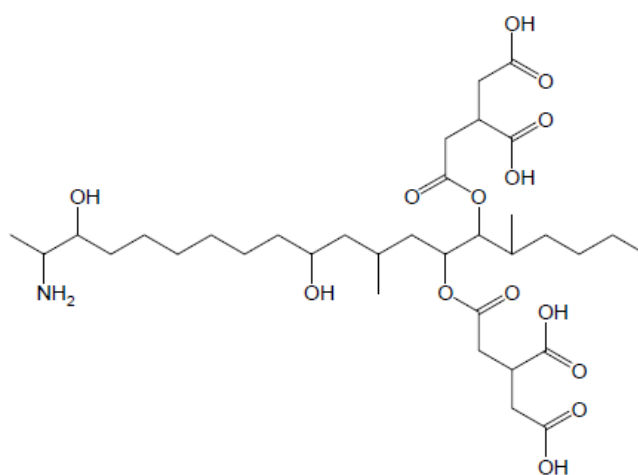
Purified fumonisins are white hygroscopic polar compounds that are readily soluble in water and aqueous solutions of polar solvents such as methanol or acetonitrile but insoluble in non-polar solvents such as chloroform and hexane (Yu *et al.*, 2014). The group B fumonisins are characterized by the presence of an amine group and they differ by lacking one of the free hydroxyl groups at either the C-10 (FB₂) or C-5 (FB₃) position. Fumonisin B₁ and FB₂ are stable during storage at -18 °C and 25 °C, respectively, but unstable above this temperature range. They are stable in acetonitrile water (1:1 v/v) at 25 °C, in methanol

at -18 °C (Yu *et al.*, 2014) and in buffer solutions over pH range of 4.8 - 9 at 78 °C (Howard *et al.*, 1998). Fumonisin is relatively heat stable; up to 120 °C and therefore survive many of the conditions used in cooking and food processing. Unless processed food is heated above 150 °C a significant drop of their concentration cannot be achieved (Bullerman and Bianchini, 2014; Milani and Maleki, 2014).



Fumonisin B₁ (C₃₄H₅₉NO₁₅)

Fumonisin B₂ (C₃₄H₅₉NO₁₄)



Fumonisin B₃ (C₃₄H₅₉NO₁₄)

Source: Tamura *et al.* (2015).

Figure 4: Chemical structure of fumonisins

2.3.2 Absorption, distribution and excretion of fumonisins

Studies where animals such as pigs, rodents, hens, turkey and cow were involved showed that after ingestion, fumonisin B₁ and B₂ are rapidly absorbed from the gastro-intestinal tract. Ingested fumonisin is distributed, quickly cleared from the blood and eliminated, with the majority excreted un-metabolized or partially hydrolyzed in faeces and urine (Norred *et al.*, 1993; Shephard *et al.*, 1999; IARC, 2002). The highest concentration of absorbed fumonisins is retained in liver and kidney (Riley and Voss, 2006). Although these studies demonstrated a quick elimination of ingested FB₁ from circulation, efforts have been made to monitor free fumonisins in human physiological samples such as urine (Shetty and Bhat, 1998; Gong *et al.*, 2008b; Ediage *et al.*, 2013), plasma (Shephard *et al.*, 1992), faeces (Chelule *et al.*, 2000, 2001) and hair (Sewram *et al.*, 2003). Findings from these studies therefore suggest the possibility for using free FB₁ as a human exposure biomarker (Xu *et al.*, 2010). Human studies indicated that urinary FB₁ half life is only about 48 hours (Riley *et al.*, 2011) and urine excretion constitute a very small amount of ingested fumonisin (Xu *et al.*, 2010; Van der Westhuizen *et al.*, 2011a; Torres *et al.*, 2014c; Riley *et al.*, 2015). A controlled human study involving adult population consuming maize of known concentration of fumonisins found that only 0.5% of ingested FB₁ is excreted in urine. This study further revealed that only FB₁ was detected in urine implying that there is lower absorption of FB₂ and FB₃ compared to FB₁ (Riley *et al.*, 2012).

2.4 Health Effects of Fumonisins

2.4.1 Disruption of sphingolipid metabolism

Fumonisins are structurally similar to the sphingoid bases, sphinganine and sphingosine, which are components of the sphingolipid molecules (Heidtmann-Bemvenuti *et al.*, 2011). This structural similarity explains the ability of the toxins to disrupt sphingolipid metabolism or inhibit the function of sphingolipids (Merrill *et al.*, 2001; Riley *et al.*, 2001;

Heidtmann-Bemvenuti *et al.*, 2011). Fumonisin B₁ disrupts ceramide synthase, the major enzyme in the sphingolipid biosynthesis pathway, thereby resulting in inhibition of the signalling pathways and cell functions of the sphingoid bases and complex sphingolipids (Dragan *et al.*, 2001; Merrill *et al.*, 2001). The sphingolipids play important roles in membrane and lipoprotein structure, cell-to-cell communication, interaction between cells and extracellular matrix and regulation of growth factor receptors (Soriano *et al.*, 2005). Inhibition of ceramide synthase by fumonisins results in inhibition of the production of ceramide and consequently the complex sphingolipids containing ceramide (Merrill *et al.*, 2001). It also causes sphinganine levels to accumulate and sphingosine levels to decrease (Merrill *et al.*, 2001). The disruption of sphingolipid metabolism lead to changes in sphinganine to sphingosine ratio, with increased sphinganine tissue concentrations, which correlates with in vivo toxicity and carcinogenicity of fumonisin (Riley *et al.*, 2001). Because sphingolipids are involved in diverse aspects of cell regulation, disruption of their metabolism may be responsible for the mechanisms of their toxicity and the carcinogenicity of fumonisins (Merrill *et al.*, 2001; Marasas *et al.*, 2004), as shown in experimental animals (Voss *et al.*, 2002).

2.4.2 Oesophageal and liver cancer

Ecological studies had identified chronic exposure to fumonisins as risk factors for high incidence of oesophageal cancer in subsistence communities where fumonisin contamination in maize was prevalent such as the former Transkei region of South Africa (Rheeder *et al.*, 1992), China (Yoshizawa *et al.*, 1994; Sun *et al.*, 2007; Xu *et al.*, 2010), Iran (Alizadeh *et al.*, 2012) and in Southern Brazil (Van der Westhuizen *et al.*, 2003). Fumonisin exposure has also been suggested as possible risk for primary liver cancer in studies conducted in China (Ueno *et al.*, 1997; Xu *et al.*, 2010). Evaluation of FB₁ has demonstrated sufficient evidence for carcinogenic effect in experimental animals.

However, due to inadequate evidence for the carcinogenicity of fumonisin in human, the toxin has been classified as “possibly carcinogenic to humans” (IARC, 2002).

2.4.3 Neural tube defects

Fumonisins exposure has been linked with cases of neural tube defects (NTD) in infants in South Texas county (Missmer *et al.*, 2006) and South Africa (Marasas *et al.*, 2004). Association between fumonisin exposure and NTD is supported by the effect of the toxin in disruption of sphingolipid metabolism, which leads to interference with folate transport across cell membranes and therefore inhibit protective effect of folic acid against the development of NTD (Stevens and Tang, 1997; Marasas *et al.*, 2004). Fumonisins have been reported to induce NTD in experimental mice (Waes *et al.*, 2005) and therefore may play a role in NTD incidence in human populations exposed to high fumonisin levels (Bulder *et al.*, 2012). Neural tube defects are embryonic defects of the brain and spinal cord that result from the failure of the neural tube to close *in utero* (Missmer *et al.*, 2006). The most common forms of NTD are spina bifida and anencephaly (Missmer *et al.*, 2006). Findings from previous studies have demonstrated that fumonisin exposure is a risk factor for human neural tube defects and related birth defects such as craniofacial abnormalities, particularly when other risk factors such as genetic susceptibility or limited availability of dietary folate exist (Marasas *et al.*, 2004). A study conducted in border counties of Texas found a significant association between increasing levels of fumonisin exposure during the first trimester with increasing odds ratios for NTD occurrences (Missmer *et al.*, 2006). These findings suggest that interventions to address NTD should also consider the control of fumonisin exposure in human.

2.4.4 Growth faltering and acute toxicity

A linkage between fumonisin exposure through consumption of contaminated maize and childhood growth faltering has been established in a previous study (Kimanya *et al.*, 2010). The study determined that infants who were exposed to fumonisins at average levels exceeding the provisional maximum tolerable daily intake (PMTDI) of 2 µg/kg body weight (bw)/day were significantly shorter by 1.3 cm and lighter by 328 g when compared to those exposed to levels less than the PMTDI (Kimanya *et al.*, 2010). However, epidemiological investigations are needed to establish the mechanism of action and extent that fumonisins affect human health, which also depend upon the availability of reliable and robust biomarkers of exposure (Voss and Riley, 2013a). The observation on impact of fumonisin on child growth faltering is in line with animal experiments which have reported on reduced body weight and decreased food consumption in piglets as a result of exposure to fumonisin (Dilkin *et al.*, 2003). Fumonisin also play a role in immunomodulation, which has been demonstrated through the effects on antibody vaccine response and changes in cytokines levels in experimental animals exposed to fumonisin B₁ (Taranu *et al.*, 2005). In India, food born disease characterised by abdominal pain and diarrhoea has been linked with acute fumonisin toxicity in human due to ingestion of contaminated maize and sorghum (Bhat *et al.*, 1997).

2.5 Malnutrition During Childhood

Malnutrition is defined as poor nutritional status. The term refers to both under-nutrition caused by inadequate supply of nutrients to the body and over-nutrition, which is the result of excess supply of nutrients (UNICEF, 2009). Under-nutrition is a significant problem in developing countries (De Onis *et al.*, 2012). The causes of malnutrition are categorised into three levels; immediate, underlying and basic. Inadequate dietary intake and diseases (severe and repeated infections) are the major immediate causes of malnutrition in Africa

(UNICEF, 1998). Food insecurity, inadequate maternal and child care practices, inadequate health care services and unhealthy environments have been identified as the major underlying factors contributing to malnutrition. Inadequate resources due to poverty is the basis of all those factors, because of its direct negative impact on the ability to meet the human basic needs at all of these levels (UNICEF, 1998).

Improved nutrition in the early period of life is fundamental and the essential foundation for lifelong human achievement in aspects of health, mortality, mental and physical development, education and economic productivity (Black *et al.*, 2013). Infants and young children are particularly vulnerable to nutritional deficiency because they have higher nutritional requirements for growth and development (Blössner and de Onis, 2005). Malnutrition leads to increased morbidity and mortality (De Onis *et al.*, 2004) and negatively impacts a child's physical, psychological and intellectual development in both the short- and long-term and compromises economic achievements during adulthood (Victora *et al.*, 2008). The period from conception to two years is considered as a critical window of opportunity for preventing under-nutrition since the impact of malnutrition is most severe and irreversible damage can occur to physical growth and cognitive development during this period (UNICEF, 2009; Victora *et al.*, 2010). It is therefore significant to assess nutritional status, intervene and prevent malnutrition during this critical time period.

2.6 Early Childhood Exposure to Aflatoxin and Fumonisin

The major route of early childhood exposure to mycotoxins is through ingestion of contaminated cereal based food and also through contaminated breast milk (Pitt *et al.*, 2012; Ghiasian and Maghsood, 2012). Childhood exposure to mycotoxins can occur at various stages; starting during foetal development (pregnancy) (Groopman *et al.*, 2014),

breast feeding, complementary feeding and consumption of family food (Khlungwiset *et al.*, 2011).

Available information, particularly for aflatoxins demonstrates that this exposure can start during foetus development (*in utero*) if a pregnant woman consumes contaminated food. The toxin is able to cross the placenta barrier and enter into the foetus (Abdulrazzaq *et al.*, 2004; Partanen *et al.*, 2010). Aflatoxin metabolites have been detected in blood samples from women during pregnancy (Turner *et al.*, 2007; Shuaib *et al.*, 2010; Yard *et al.*, 2013; Castelino *et al.*, 2014), at delivery (De Vries *et al.*, 1989) and in cord blood samples of neonates (De Vries *et al.*, 1989; Abdulrazzaq *et al.*, 2004). After a child is born, the exposure to mycotoxins may continue during early infancy as a result of consumption of breast milk contaminated with AFM₁ (Khlungwiset *et al.*, 2011; Magoha *et al.*, 2014a) and fumonisin B₁ (Magoha *et al.*, 2014b), which result from dietary exposure of breast feeding mother to aflatoxins and fumonisin. Contamination of breast milk with AFM₁ has been reported in several countries such as Egypt (Polychronaki *et al.*, 2007b), Sudan (Elzupir *et al.*, 2012) and Turkey (Uyar *et al.*, 2014) and recently in Tanzania (Magoha *et al.*, 2014a). Aflatoxin M₁ in milk is less toxic than the parent aflatoxin B₁ found in food, but still has cytotoxic effect (IARC, 2002). Detection of aflatoxin in pregnant mothers, cords blood of neonates and in breast milk suggests that measures to prevent children from the exposure should also consider preventing the maternal exposure, which is a possible potential source of child exposure at an early age. Consequently, the exposure can be extended to the complementary feeding period due to mycotoxin contamination in complementary food ingredients mainly maize and groundnuts. Exposure levels have been reported to increase with age due to child transition from breast feeding to consumption of complementary foods. It has further been reported that children who rely solely on complementary foods have demonstrated higher levels of aflatoxin exposure compared to children who are

breastfed (Gong *et al.*, 2003, 2004). Aflatoxin exposure in children during complementary feeding has been documented in various countries such as Gambia (Turner *et al.*, 2003, 2007) Uganda (Asiki *et al.*, 2014), Togo and Benin (Gong *et al.*, 2002, 2003, 2004) and Egypt (Shouman *et al.*, 2012). Exposure of young children to fumonisin during their complementary feeding period has been documented from a biomarker study conducted in Cameroon (Ediage *et al.*, 2013).

2.7 Determinants of Exposure to Aflatoxin and Fumonisin

The magnitude of human exposure to mycotoxins depends on the level of toxin contamination in foods and the quantity of the food that is consumed (Shephard, 2008). The greater susceptibility of maize to both aflatoxin and fumonisin contamination as well as groundnuts to aflatoxin contamination coupled with high consumption of these two food products are the major sources of exposure of human to the mycotoxins (Strosnider *et al.*, 2006). Mycotoxins or their metabolites may be carried over into animal products when animals are fed on contaminated feeds. In view of this, human exposure to the toxins can further occur from consumption of animal products such as milk, meat, eggs and their associated products (Tchana *et al.*, 2010; Pitt *et al.*, 2012). Lack of dietary diversity and food insufficiency further contribute substantially to the susceptibility of individuals and communities to mycotoxins exposure (Strosnider *et al.*, 2006). The seasonality or timing of samples collection (Wild *et al.*, 2000b; Ghiasian *et al.*, 2007) and place of residence (Gong *et al.*, 2002, 2003; Adejumo *et al.*, 2013; Ediage *et al.*, 2013) influence the levels of mycotoxin exposure in populations. In Gambia, higher exposure levels were reported during dry seasons, which were partly due to consumption of incorrectly stored groundnuts, with higher accumulated aflatoxin levels (Wild *et al.*, 2000b). Higher exposure levels were reported among individuals in rural than peri-urban areas and this was explained as due to consumption of more varied diet and less reliance on groundnuts in peri-urban than in rural

areas. A study in Togo and Benin demonstrated that aflatoxin exposure levels were lower in children aged less than one year and increased with age until at age of 2 - 3 years old at which the exposure levels reached a plateau (Gong *et al.*, 2003). This association was not significant when adjusted for agro-ecological zone, socio-economic status and complementary feeding status suggesting that increased age is indicator of increased reliance on complementary foods, which in turn is associated with increased exposure if complementary food is contaminated.

2.8 Overview of Biomarkers of Human Exposure to Mycotoxins

Biomarker of exposure refers to measurement of the specific agent of interest, its metabolites, or its specific interactive products in a body compartment or fluid, which indicates the presence and magnitude of current and past exposure (Kensler *et al.*, 2011). Biomarkers of exposure include the parent compounds, the metabolites of the toxin and the products of the reaction of the toxin or its metabolites with molecules such as protein and DNA in human blood, urine or tissue samples (Routledge and Gong, 2011). In this perspective, use of biomarkers provides information on quantification of the parent toxin compound or its biotransformation products in body fluids (i.e biomarkers of exposure) or allows biological responses due to exposure (i.e biomarkers of effect) (Miraglia *et al.*, 1996). Determination of the mycotoxins or its metabolites in a biological medium such as blood or urine is one of biological methods used to assess exposure and or risk to health (Silins and Högberg, 2011). The levels determined may reflect exposure over different time periods, depending on the kinetics of the toxin, the medium involved and the time of sampling (Needham *et al.*, 2005).

2.8.1 Potential of biomarkers in assessment of human exposure to mycotoxin

Mycotoxins are associated with various health effects in human population. In order to evaluate human health impact due to mycotoxins ingestion, accurate methodology for assessment of exposure and evaluation of effectiveness of intervention strategies are important and can be achieved by application of biomarkers of mycotoxins exposure (Miraglia *et al.*, 1996; Shephard *et al.*, 2007, 2013b; Leong *et al.*, 2012).

Application of biomarkers method is a useful for establishing oral dose from food intake, the internal dose as demonstrated by biologically active compound as well as the dose-response relationship (Miraglia *et al.*, 1996). Dietary assessment is the most common methodology, which has been extensively used in evaluation of mycotoxins exposure based on estimation of food contamination level combined with food intake data and measurement of individual body weight (Warth *et al.*, 2012). However, use of dietary assessment seems to be unreliable and less helpful in estimation of mycotoxins exposure at individual level as compared to biomarker assessment method (Shephard *et al.*, 2007).

Exposure assessment by using biomarkers gives better estimate of human exposure to mycotoxins (Paustenbach and Galbraith, 2006; Turner, 2013a, b). The biomarker method takes into account variations associated with food such as levels of toxin contamination and distribution, dietary composition, food preparation methods, food sampling procedures, accuracy of food analytical methods and assessment of dietary intake. Likewise biomarker takes in account variations in absorption, distribution, elimination and excretion of ingested toxin (Shephard *et al.*, 2007). It is on these grounds that evaluation of mycotoxins in biological fluid has been emphasised as the most promising way for correct indication of toxins intake from diet and risks associated with the toxins (Miraglia *et al.*, 1996). While biomarkers is regarded as the most suitable method for assessing exposure, it has also been

recommended to consider that even well validated exposure biomarkers do not provide an absolute assessment of exposure but rather, what they offer is improved exposure (Turner, 2013a, b). Several biomarkers have been developed for aflatoxin and fumonisin exposure measurement (Routledge and Gong, 2011). Validated exposure biomarkers for aflatoxins have been well established and these include serum aflatoxin albumin adduct, urinary aflatoxin M₁, aflatoxin-N7-guanine and urinary DNA adduct (Routledge and Gong, 2011; Turner and Pasturel, 2013a). For fumonisin, the steps for developing exposure biomarkers and validation have significantly advanced in recent years (Van der Westhuizen *et al.*, 2010, 2011a, 2013; Turner and Pasturel, 2013a). Validated exposure biomarkers for fumonisin include urinary fumonisin B₁ and sphinganine (Sa) to sphingosine (So) ratio (Shephard *et al.*, 2007; Routledge and Gong, 2011).

2.8.2 Potential biological matrices for quantification of exposure to mycotoxin

Recent development and application of biomarker in aflatoxin and fumonisin research, have been summarised extensively by Routledge and Gong (2011). In biomarker applications, factors such as routes of exposure, metabolism and volatility of the toxins determines the type of human sample that can be collected and analysed (Kensler *et al.*, 2011). The most frequent biological matrices used in quantification of mycotoxin exposure are blood, urine, milk, faeces (Shephard *et al.*, 2007; Leong *et al.*, 2012), while hair, nails, sputum or exfoliated cells have been observed as potential matrixes to consider. However, due to some practical and ethical considerations, blood and urine samples have generally been collected (Paustenbach and Galbraith, 2006). For accurate application in exposure assessment, stability of the biomarker during storage needs to be considered as well (Kensler *et al.*, 2011). Aflatoxin-albumin adducts in human sera has been proved to be stable for at least 25 years when stored at -20 °C (Scholl and Groopman, 2008). Due to covalent binding of aflatoxin to albumin in peripheral blood, the levels of adducts formed is

assumed to reflect exposure to aflatoxin over the preceding 2 - 3 months, based on the half-life of albumin, hence represents chronic exposure (Wild *et al.*, 1986; IARC, 1993). This exposure biomarker has demonstrated significant correlation with aflatoxin intake both in animal and human research (Gan *et al.*, 1988; Wild *et al.*, 1992). On the other hand, a controlled human study by Riley *et al.*, (2012) on relationship between FB₁ intake from maize based food and urinary excretion found excretion of UFB₁ to be highly variable, peaking soon after consumption and decreased rapidly after consumption was stopped. Within 5 days after consumption was stopped, FB₁ was not detected in urine. This suggests that FB₁ is rapidly absorbed from gastro-intestinal tract and therefore UFB₁ reflects a recent exposure (Riley *et al.*, 2012). Fumonisin B₁ concentration in the urine has been demonstrated to remain stable when urine is stored frozen at -20 °C for one at least one year (Riley *et al.*, 2012). Consequently, methods for assessing exposure of human to the toxin has been developed, validated and applied (Gong *et al.*, 2008b; Ediage *et al.*, 2013).

2.9 Strategies for Managing Exposure to Mycotoxins

Presence of sufficiently high levels of mycotoxin in food pose a significant food safety risk, health hazard and negative economic impact (Zain, 2011). The economic impact of mycotoxins on humans can be assessed by several ways such as loss of human life, health care costs, regulatory costs and research cost focusing on alleviation of impact and severity of the mycotoxin problem (Wagacha and Muthomi, 2008). In view of these perspectives, management of mycotoxins contamination in food can help to reduce exposure and therefore protect health, reduce financial burden on health care, and promote food security and trade (Strosnider *et al.*, 2006; Wu, 2014).

2.9.1 Pre-harvest strategies

The implementation of good agricultural practices can reduce mycotoxins and fungal infection of food crops in the field before harvest and consequent contamination of harvested produce (Strosnider *et al.*, 2006; Wagacha and Muthomi, 2008; Eeckhout *et al.*, 2013; Pitt *et al.*, 2013). Pre-harvest interventions include crop rotation, pest management, reducing drought stress, using crops which are resistant to drought, as well as pest, disease and fungal control (Zaki *et al.*, 2012; Eeckhout *et al.*, 2013; Munkvold, 2014). Pests infestation makes maize plants more susceptible to fungal invasion and hence to mycotoxin contamination (Chulze, 2010). Application of genetic modification such as incorporating into the maize plant the gene from the bacterium *Bacillus thuringiensis* (*Bt*) is known to be an important potential tool for insect pests protection, hence effective in reducing concentrations of mycotoxins in maize (Ostrý *et al.*, 2015). The *Bt* maize is a commonly grown genetically modified crop, carrying the *Bt* gene for production of a toxin, that is used as conventional insecticide in agriculture (Ostrý *et al.*, 2015) and is safe for human consumption (WHO, 2014). Effective measures to combat pests have a positive effect in reducing mycotoxin levels (Ostry *et al.*, 2010). Numerous studies have reported a significant reduction in pest damage and mycotoxin levels in maize hybrids with the *Bt* gene compared to non-*Bt* maize hybrids (Williams *et al.*, 2010; Abbas *et al.*, 2013; Ostrý *et al.*, 2015). However, the *Bt* seed maize has shown to have a greater economic impact on *Fusarium* toxins such as fumonisins than on aflatoxins (Díaz-Gómez *et al.*, 2015).

Basic measures such as removal of infected debris from previous harvest help in minimizing infection and infestation of produce in the field (Hell and Mutegi, 2011; Olanya *et al.*, 1997). Timely harvesting has also been reported to reduce fungal infection of crops before harvest and consequent contamination of mycotoxins in harvested produce (Zain, 2011). Reduced levels of aflatoxin has been demonstrated in early harvested and

shredded groundnuts compared to delayed harvesting (Rachaputi *et al.*, 2002). Other strategies to reduce mycotoxins include use of biological control such as atoxigenic fungi that can out-compete the mycotoxins producing strains in the field (Medeiros *et al.*, 2012; Aliabadi *et al.*, 2013; Tran-Dinh *et al.*, 2014; Chulze *et al.*, 2015). Appropriate use of chemicals such as fungicides and pesticides help in reducing fungal infection and insects infestation of crops thereby reducing crop susceptibility to mycotoxins contamination (Scarpino *et al.*, 2015). However, due to concerns of environment and food safety issues, use of fungicides is being discouraged (Strosnider *et al.*, 2006).

2.9.2 Post harvest strategies

Post harvest handling and processing measures such as rapid and proper drying of harvested agricultural products to reduce moisture content before storage is critical in reducing insect infestation, fungal growth and proliferation (Strosnider *et al.*, 2006; Zain, 2011). Adequate drying, sanitation, ventilation, elimination of insect and pests activity during handling, storage and transportation of crops are important undertakings that can reduce mycotoxins contamination and subsequent exposure (Zain, 2011; Zaki *et al.*, 2012).

An intervention study on proper drying and storage of groundnuts achieved 60% reduction in mean serum AF-alb levels in the human intervention group, suggesting that simple and cheap methods can have significant impact in reducing human exposure to aflatoxins (Turner *et al.*, 2005; Strosnider *et al.*, 2006). Physical treatment such as sorting and disposal of visibly mouldy, discoloured and damaged grains and nuts, winnowing and washing of maize grains are reported to be effective methods of reducing levels of mycotoxin contamination and exposure (Van der Westhuizen *et al.*, 2011b; Fandohan *et al.*, 2005a). Studies on the distribution of aflatoxin or fumonisin in grains showed that a major portion (up to 80%) of the toxin is often associated with the small and shrivelled

seed as well as mouldy and stained grains (Fandohan *et al.*, 2005a; Turner *et al.*, 2005). Fumonisin are more concentrated on the outer layer and germ of a maize kernel than in the endosperm (Duncan and Howard, 2010), therefore removal of the kernel's outer parts by mechanical process such as de-hulling can significantly reduce this toxin. Steeping of contaminated kernels by dipping the whole kernel in water for 6 to 48 hours has also been proven to leach fumonisin into water and reduce contamination (Canela *et al.*, 1996).

Nixtamalisation (alkaline cooking), a food processing method in which the whole kernel maize is cooked in a lime solution (calcium hydroxide) to prepare maize products such as *masa* and *tortillas* effectively reduces the concentration of fumonisin B₁ (Voss *et al.*, 2013b; De Girolamo *et al.*, 2016). The method is commonly used in Mexico and Central America (Pietri and Bertuzzi, 2012). Other food processing practices which can potentially reduce mycotoxin contamination in food include fermentation (Okeke *et al.*, 2015), extrusion (Zheng *et al.*, 2015; Suman and Generotti, 2015) and ammoniation (Atanda *et al.*, 2012).

2.9.3 Individual based intervention method

Use of enterosorption technique has been applied in management of mycotoxin exposure in animals (Weaver *et al.*, 2013; Di Gregorio *et al.*, 2014; Mitchell *et al.*, 2014; Maki *et al.*, 2016) and in human (Robinson *et al.*, 2012). Enterosorption refers to use of mineral adsorbents such as processed calcium montmorillonite in mycotoxin contaminated food or feed in order to bind the toxins and prevent their uptake from the gut (Turner, 2014). This measure has been proven to be protective against animal and human exposure because of its affinity to bind aflatoxins and make them unavailable for absorption in the body (Mitchell *et al.*, 2014; Maki *et al.*, 2016). The technique also prevents human exposure to toxins that might have been carried over from animal products. However, impact from

possible binding of adsorbent agents to other nutrients along with mycotoxins should also be taken into consideration (Juma *et al.*, 2015).

Use of chemoprotection technique has been applied in management of mycotoxin effects. The method involve the use of chemical compounds (such as oltipraz, chlorophyllin) or dietary intervention (such as use of antioxidants) to alter susceptibility of human to carcinogens by increasing detoxification processes or prevent the production of the epoxide, which is the active toxic form of aflatoxin and hence reduce the damage to the cells (Wang *et al.*, 1999; Tang *et al.*, 2008; Turkez *et al.*, 2012; Turner, 2014). Food processing strategies mainly alkaline treatment of maize (nixtamalization) can also offer chemoprotective benefits (Rojas-García *et al.*, 2012; Acosta-Estrada *et al.*, 2015; Reynoso-Camacho *et al.*, 2015). However, enterosorption and chemoprotection techniques are expensive and therefore difficult to implement in poor communities, but also their safety, efficacy and acceptability is uncertain (Strosnider *et al.*, 2006; Robinson *et al.*, 2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of Study Areas

This study was conducted at Nyabula, Kigwa and Kikelelwa villages from geographically distant regions of Iringa, Kilimanjaro and Tabora, respectively (Fig. 5). The road distance from Iringa to Kilimanjaro is 769 km, Iringa to Tabora is 839 km and Tabora to Kilimanjaro is 741 km (TANROADS, 2009) and their global positioning coordinates are specified under description of each region in the next sections. These three regions are among the 25 regions of Tanzania Mainland and were selected for the study because each one represents characteristics of an agro-ecological zone, which is different from the other regions. In view of this, the regions are likely to vary in terms of magnitude of contamination of aflatoxins and fumonisins in food and subsequent exposure of humans to the toxins. Another selection criteria was based on findings from previous studies conducted in these regions, which demonstrated that maize was highly contaminated with aflatoxins and fumonisins (Kimanya *et al.*, 2008a). The same regions therefore were followed up to examine the extent of exposure to the mycotoxins among consumers especially children.

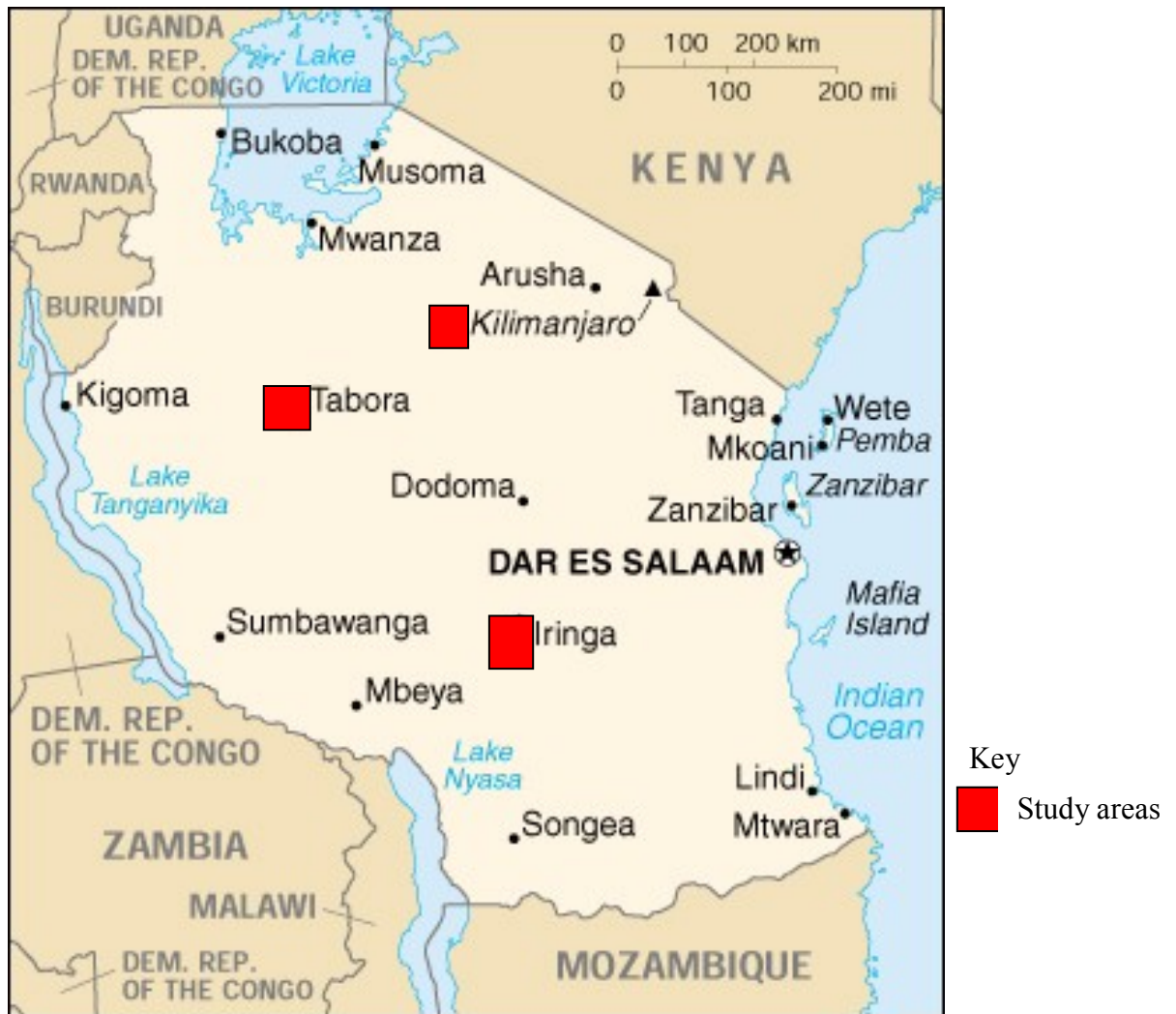


Figure 5: Tanzanian map showing the study areas

3.1.1 Iringa region

Iringa region is located in the Southern Highlands zone, at latitudes $6^{\circ}55'$ and $10^{\circ}30'$ South of the Equator and between longitudes $33^{\circ}45'$ and $36^{\circ}55'$ East of Greenwich. The region is divided into five districts namely Iringa district council, Mufindi, Iringa municipal council, Kilolo and Mafinga (URT, 2013). The study was conducted at Nyabula village in Iringa district council, which is one of the five districts in the region. The District extends between latitudes $7^{\circ}00' - 8^{\circ}30'$ South and longitudes $34^{\circ} - 37^{\circ}$ East. The average annual rainfall varies between 500 - 2700 mm and temperature is typically 15°C , ranging between $15^{\circ}\text{C} - 30^{\circ}\text{C}$. Iringa district council has a population of 254 032 people (URT, 2013). The dominant

ethnic groups in the district are Wahehe and Wabena (Iringa District Council Socio-economic Profile, 2013). The region's largest economic sector is agriculture and maize is major staple food crop and cash crop for the majority of the population. The region is categorised as one of the major maize producers in the country. Maize production in Iringa district council is 68 360 t (URT, 2006) and estimated maize consumption at Iringa region is 549.5 g/person/day (Smith and Subandoro, 2007). Other important food crops are potatoes, sunflower seeds, beans, cowpeas, fruit and vegetables. Likewise, livestock farming is practiced with dairy cattle, sheep, goat, pig and poultry (Iringa District Council Socio-economic Profile, 2013).

3.1.2 Kilimanjaro region

Kilimanjaro region is located in the North Eastern part of Tanzania Mainland. The region lies between latitudes 2°25' and 4°15' South of the equator and between longitudes 36°25'30" and 38°10'45" East of Greenwich. Administratively, the region is divided into six districts namely Hai, Rombo, Mwanga, Same, Moshi and Siha. Kikelelwa village where the study was conducted is at Rombo district. The district is located on the slope of Mount Kilimanjaro between latitude 3°09' South and longitude 37°33' East. The average temperature ranges between 18 °C - 20 °C. In terms of climate, the district is classified as tropical savanna but due to influence of Mount Kilimanjaro, the climate varies considerably. The district receives a mean rainfall of about 500 mm per annum. The rainfall pattern is bimodal, unreliable, erratic and poorly distributed. The district had a population of 260 963 in 2012 (URT, 2013). The main ethnic group in the district is the Chagga (Rombo District Socio-economic Profile, 2013). Small scale agriculture is the main economic activity in the area comprising about 90% while business and employment covers 7% and 3% of the economic activities, respectively. Food crops grown at Rombo district are bananas, maize, beans, round potatoes, yams, cassava, sweet potatoes, finger

millet, sorghum, groundnuts, cowpeas, pigeon peas fruits and vegetables (Rombo District Socio-economic Profile, 2013). Maize and groundnuts production at the district is 10 410 t and 160 t, respectively (URT, 2006) and estimated maize consumption at the region is 213.3 g/person/day (Smith and Subandoro, 2007). Dairy cows are rarely found in the district due to lack of fodder but small ruminants like sheep, goats, poultry, pigs and rabbits are raised (Rombo District Socio-economic Profile, 2013).

3.1.3 Tabora region

Geographically, Tabora region is found in the mid-western part of the Tanzania Mainland on the Central African Plateau between latitude 4° and 7° South of equator and longitude 31° and 34° East of Greenwich. The region's daily mean temperature is around 23 °C. The region is divided into seven districts namely Kaliua, Tabora Municipal Council, Sikonge, Urambo, Nzega, Igunga and Uyui. Kigwa village where the study was conducted is located at Uyui district which lies between latitudes 05°04' and 06°15' South of the Equator and between longitudes 32°15' and 32°00' East of Greenwich and occupies a total surface area of 13 453 sq. km. The district receives rainfall of between 750 mm and 950 mm annually. The main ethnic groups are Nyamwezi and Sukuma (Uyui District Socio-economic Profile, 2008). According to the 2012 Population and Housing Census, the district had 396 623 people (URT, 2013).

The main economic activities of the region are agricultural production and livestock farming. The dominant food crop is maize followed by cassava, rice and sorghum. Other food crops are groundnuts, sunflower, sweet potatoes and legumes. Tobacco, cotton, groundnuts and sunflower are the cash crops of economic importance. Livestock kept in the district are cattle, goats, sheep and poultry (URT, 2005). Maize and groundnuts production

at Uyui district is 17 220 t and 6600 t, respectively (URT, 2006) and estimated maize consumption at Tabora region is 647.2 g/person/day (Smith and Subandoro, 2007).

3.2 Subjects for the Study

A total of 166 seemingly healthy children (i.e without any obvious signs or symptoms of disease), at age between 6 and 14 months were randomly recruited from the study areas and enrolled in this study. One child was selected per family, based on the birth registration and child's health clinic records at village dispensary or health centre. The age of recruitment to participate in the study was selected on the basis that it was age period when the children were very likely to be exposed to aflatoxins and fumonisins from contaminated ingredients of complementary foods, particularly maize and groundnuts.

3.3 Sample Size Calculation

Sample size was calculated based on previously established formula (Naing *et al.*, 2006) as:

$$n = [Z^2 P (1 - P)] / d^2 \text{ whereby;}$$

n = sample size

Z = Z statistic for a level of confidence (1.96 at confidence level of 95%)

P = 0.1, based on earlier study by Kimanya *et al.* (2008a), which found that 10% of maize samples were contaminated with both aflatoxins and fumonisins

d = precision (0.05)

Therefore; $n = 1.96 \times 1.96 \times 0.1(1 - 0.1)/(0.05 \times 0.05) = 138$. Assuming 20% possible attrition rate, sample size was adjusted to $n = 138 + (20/100 \times 138) = 166$.

While taking into account the total sample size for the study, a sample size for each village was determined by a village sampling frame.

3.4 Study Design

This was a longitudinal study, whereby enrolled subjects were studied at three main survey periods. The first survey was conducted during maize harvest season since it was expected that during that season, children will largely be consuming the newly harvested maize (harvest I). The second survey took place after 6 months from the first survey, during which stored maize from harvest I was consumed. The final survey was conducted at 12 months after the first survey, which was another season when newly harvested maize was consumed (harvest II). For purpose of simplicity and consistence, the three surveys will be referred to as “at recruitment”, “6 months after recruitment” and “12 months after recruitment” to represent the surveys conducted at the first season of maize harvest, the season during which stored maize was consumed and the second season of maize harvest, respectively. This study was so designed to enable exploration of seasonal and geographical variations in aflatoxin and fumonisin exposure, to monitor and track child growth, exposures and association between the exposure and growth.

3.5 Ethics Clearance

Ethical approval for the study was granted by the National Institute of Medical Research in Tanzania, and the University of Leeds, UK. Informed written consent of participation in the study was obtained from mothers or care providers of each participating subject during an information session about the nature and details of the study. Mothers or child care providers who agreed to participate in the study signed a note of informed consent (Appendix 1).

3.6 Data Collection

During each of the three surveys, each family of participating child was visited for data collection. Data for the study were collected through face to face interviews of child's

mother or care provider by using a structured questionnaire, through observation, taking anthropometric measurements of child weight and length and collection of samples of urine and blood. Before the questionnaire was administered, it was first pre-tested to ten mothers in the study areas and revised accordingly. Data collection was done with support from field assistants, who were pre-trained on how to collect the data.

3.6.1 Children demographic and socio-economic data

Children's information on birth date, birth weight, sex and immunisation record were obtained from their health clinic cards. For each child, a structured questionnaire (Appendix 2) was administered to his/her mother or child care provider to collect information about parents' education levels, occupation, marital status, number of children and family size. Data was also obtained on maize processing methods such as dehulling and source of maize since these practices are potential determinants of magnitude of mycotoxins contamination in food and the subsequent exposure through consumption.

3.6.2 Household socio-economic status

For each child, socio-economic status (SES) of his/her household was assessed based on observation of type of family house in terms of building material used for floor (soil/earth or cement), wall (soil/mud, mud brick, timber, burnt bricks or concrete blocks) and roof (thatched roof or iron sheets). It was expected that households using soil or grass as building materials for houses are considered as poorer when compared to those using cement/concrete bricks and iron sheets since the former can easily obtain the locally available building materials without much cost. In grouping households according to their SES, the materials used for building the house were assigned own determined scores in such a way that for wall, one (1) was assigned for mud wall, one point five (1.5) for mud brick wall, two and half (2.5) for timber or burnt bricks wall and five (5) for concrete

blocks wall. For roof materials, one (1) was assigned for thatched roof and four (4) for iron sheets roof. For floor, one (1) was assigned for soil floor and five (5) for cement floor. Combined scores from wall, roof and floor were categorised into three groups, which enabled households to be grouped into low, medium and high SES. The approach of using wealth index or assets as estimate of expenditure and income is recommended especially in developing countries where it is difficult to get reliable estimates of income (Bawah and Zuberi, 2004). In addition, use of household durable assets such as ownership of a house has been considered as good measure of long-term wealth, which may have impact on long term nutritional deficiency such as stunting (Fotso *et al.*, 2012).

3.6.3 Child feeding practices and estimation of food intake

Information about breastfed status and age at which complementary foods were introduced to infants was obtained through interviews using a questionnaire. During each survey period, a repeat 24-hour dietary recall questionnaire was administered to obtain data on details of type, ingredients and quantities of foods given to a child in two days. This approach has been previously validated for similar study population by other researchers in Tanzanian children (Mamiro *et al.*, 2005; Kimanya *et al.*, 2009). During home visits, each mother or child care provider was requested to show the quantity of each food that the child consumed for each feeding during the previous day. For foods in liquid form such as thin porridge, the respondent was asked to estimate the amount given to the child by using water in utensil (cup or bowl) that is normally used in feeding the child. The water was transferred to a graduated bottle and the quantity was recorded as amount of the food the child consumed. For foods which were in thick form, their quantities were estimated based on number of table spoonful portions (assuming one table spoonful is equivalent to 10 g) that were consumed by a child. The mean of the estimated intake for the two recall days was used as the consumption data for each type of food consumed by a child. A food

frequency questionnaire was administered to assess frequency of maize and groundnuts (i.e. foods most prone to mycotoxin contamination) intake in the previous week before the survey.

3.6.3.1 Estimation of protein and energy intakes

Protein and energy intake levels were calculated based on dietary information obtained from the 24-hour dietary recall. Total intake for each food consumed by a child per day was quantified and the respective levels of protein and energy consumed from each type of food were calculated based on their respective levels established per 100 g of food of a similar recipe provided in the Tanzania Food Composition Tables (Lukmanji *et al.*, 2008). Protein and energy intake levels per day from all types of food consumed was calculated by using the formula $X_i = (Y_i \times Z_i)/100$ whereby;

X_i = amount of energy (kcal) or protein (g) intake from food “i”

Y_i = total weight (g) of a particular food “i” consumed per day

Z_i = level of protein or energy content provided by 100 g of edible weight of food “i” as provided in the Tanzania Food Composition Tables.

3.6.3.2 Estimation of quantities of maize intake by children

Maize is a potential source of aflatoxin and fumonisin exposure in humans. The common maize based foods eaten by infants and young children in the study areas were mainly in the form of thin porridge and stiff porridge. The equivalent weight of maize intake per day was extracted from the 24 hour recall information data on estimated quantities of thin and stiff porridges eaten by each child. The formula used to calculate quantities of the maize intake from thin and stiff porridge were $17\% \times$ quantity of thin porridge consumed and $36\% \times$ quantity of stiff porridge consumed, respectively. The factors 17% and 36% were based on proportions of maize flour in the respective porridges, obtained through optimised

in house experiment in which it was established that the average proportion of maize flour in stiff porridge was 36% while the proportion in thin porridge was 17% based on local recipe. These factors were further verified after these foods were oven dried in the laboratory to constant weight, followed by the adjustment of the dried matter to the moisture content of maize flour, which is 13%. For each child, quantity in dry weight of maize intake per day adjusted for body weight was calculated and expressed in g/kg bw/day.

3.6.3.3 Consumption of groundnuts

Data on groundnut consumption was collected since this food crop is a potential source of aflatoxin exposure. During each survey, respondents were requested to recall if during the previous week before the survey, a child received complementary foods that contained groundnuts and number of days per week in which groundnuts based foods were consumed.

3.6.4 Anthropometric measurements of children and computation of growth indices

Anthropometric measurements of body weight and recumbent length were taken by trained field workers according to the World Health Organisation (WHO) standardised procedures using calibrated instruments (WHO, 2006). All the measurements were repeated to obtain three readings for each child.

3.6.4.1 Weight measurement

The weight of each child was measured with a portable spring scale (Salter model 235 6M). The scale was hanged from a secured place at eye level of the person taking the measurement by using a strong piece of rope. A pair of empty weighing pants was attached to the hood of the scale and the scale was adjusted to zero. A mother or child care provider

was asked to undress the child as much as possible to leave her/him with minimum or light clothing and to remove from the child all heavy items such as shoes, socks, jewelry and diaper/nappy. Child's legs were grasped and pulled through the holes of the weighing pants. While holding the child, the scrap of the pants was attached to the hook of the hanging scale and the child was gently lowered and allowed to hang freely without touching anything. The weight measurement was read on stabilisation of the scale pointer and recorded to the nearest 100 g.

3.6.4.2 Length measurement

Recumbent length was measured using the length measuring board, which had fixed head rest and a movable foot piece [SECA 416 infantometer (SECA, UK)]. Before taking the measurement, the board was positioned on a flat and stable table surface. A mother or child care provider was requested to remove child's shoes, socks and hair ornaments. For hygiene and comfort of a child, length board was wiped and covered with paper towel before a child was placed on it. A mother or child care provider placed the baby on the length board with face-up, with head against the fixed head piece and child's eyes looking straight up. Meanwhile, the mother was also asked to sooth and comforts the child in order to obtain good measurement. Care was taken to ensure that the child lies straight along the board, with shoulders touching the board and spine not arched or moved out of position. The person taking the measurement held down the child's legs and applied gentle pressure to the knees to straighten the legs. While holding the knees, the footboard was pulled against the child's feet, with soles of the feet placed flat against the foot piece and toes pointing upwards. The length measurement was carefully read and recorded to the nearest 0.1 cm. When the last (i.e third) survey was conducted, some of the children (n = 21) were aged 24 months and older and since they were measured in recumbent length instead of standing height, adjustment was made by subtracting 0.7 cm from each child's length. This

adjustment was made in order to convert the length into height prior to computation of growth indicator Z scores as recommended by WHO, since standing height is about 0.7 cm less than recumbent length (WHO, 2005).

3.6.4.3 Computation of growth indices and categorisation of nutritional status

Growth indices of length for age Z-score (LAZ), weight for age Z-score (WAZ) and weight for length Z-scores (WLZ) were computed using WHO Anthro software version 3.1.0. Children with LAZ, WAZ and WLZ scores below -2 standard deviation (SD) of the WHO growth reference standards were classified as stunted, underweight and wasted respectively, while children with WLZ scores more than +2 SD were considered as overweight (WHO, 2006). For each child, the respective length and weight gained during the 12 months of the study period was calculated from difference between the last and initial measurements. During each survey, child's age was calculated from the difference between date of survey when anthropometric measurements were taken and birth date.

3.6.5 Collection and handling of blood samples

In order to provide measures of exposure of children to aflatoxin, blood samples were collected for analysis of aflatoxin exposure biomarker. Each mother or child care provider was requested to bring the child at a village dispensary, health centre or hospital where blood sample was collected. During each survey, a single sample of 2 ml of venous blood from each child was collected by a qualified nurse or hospital laboratory technician in accordance to the standardised procedures. The sample was collected into a 4 ml vacuum plain tube without coagulant (BD VACUTAINER®). Plasma was separated by centrifugation at 3 000 rpm for 10 minutes at a regional or district hospital laboratory in the study areas. The obtained plasma was carefully transferred by pipette into a 2 ml sterile cryovial tube with screw lid (SARSTEDT) pre-labelled with child's identification number,

closed and immediately stored in a -20 °C freezer. From hospital laboratory at study sites, frozen samples were transported in a cold box to TFDA laboratory in Dar es Salaam, where they were maintained frozen at -20 °C. From the TFDA laboratory, the frozen plasma samples were transported on dry ice by airway to the laboratory at University of Leeds, England for analysis of aflatoxin exposure biomarker.

3.6.6 Collection and handling of urine samples

Urine samples were collected for analysis of fumonisin exposure biomarker. In order to overcome challenges associated with collection of urine sample from young children, pre-testing was done in 30 children (10 from each village) after which, appropriate modifications in urine collection procedures were made before the start of the actual collection. Mothers or child care providers were carefully trained and demonstrated on how to collect the urine sample by using paediatric urine bags and handle them as described in Appendix 3. During each survey, a child's sample of all night urine was collected by the mother/child care provider using paediatric urine bags (Hollister, USA). Two samples of urine were collected on two consecutive days in order to obtain a representative estimate of exposure. About 40 - 45 ml of collected urine was carefully transferred into a conical tube with screw lid (SARSTEDT) pre-labelled with child's identification number, closed and stored in a cold box immediately following collection at the study sites until transferred into -20 °C freezers at district or regional hospital laboratory within 6 - 8 hours. The frozen samples were further transported in ice box to TFDA laboratory in Dar es Salaam and stored at -20 °C. Just like plasma samples, urine samples were also transported in dry ice by airway to the laboratory at University of Leeds, England for analysis of fumonisin exposure biomarker.

3.7 Laboratory Analysis of Samples of Blood and Urine

Analyses of samples of blood and urine for biomarkers of aflatoxin and fumonisin exposure respectively were performed in the laboratories at Leeds Institute of Genetics, Health and Therapeutics, University of Leeds, United Kingdom.

3.7.1 Determination of aflatoxin albumin adduct levels in plasma

The biomarker of aflatoxin exposure analysed in plasma samples was AF-alb. Levels of AF-alb were determined by following the previously described method (Chapot and Wild, 1991). Detailed procedure for the analysis is described in Appendix 4. In brief, the procedure involved four major steps, which are extraction of albumin from plasma, digestion of protein, purification and Enzyme linked immunosorbent assay (ELISA) quantification of the AF-alb. Each batch of plasma was analysed with three positive controls and one negative control for the purpose of quality control. Measurements were done in quadruplicate on at least two occasions on separate days. The limit of detection for the assay was 3 pg /mg albumin.

3.7.2 Determination of fumonisin levels in urine

Free urinary fumonisin B₁ (UFB₁), a biomarker of fumonisin exposure, was measured. Analytical procedures were performed in accordance to the method previously validated by Gong *et al.*, (2008b). Details of the method are presented in Appendix 5. In brief, the frozen urine samples were thawed and centrifuged at 5 000 rpm for 15 min at 4 °C. A 10 ml aliquot of the supernatant was collected and diluted with an equal volume of distilled water and 2 ng of a stable isotope deuterium labelled FB₁ (FBd6) was added as an internal standard. The FB₁ in urine was isolated by solid-phase extraction (SPE) using Oasis^R MAX cartridge (Waters, UK). The cartridge was preconditioned with 2 ml methanol:water (1:1, v/v) after which the sample was slowly loaded at a flow rate of 1 ml per minute. The

cartridge was then washed with 2 ml of 5% ammonium hydroxide in water and 2 ml of 100% methanol consecutively. Fumonisin B₁ was eluted with 2 ml of 2% formic acid in methanol at a flow rate of 1 ml per minute. The eluate was dried under vacuum using a Savant Speed Vac and reconstituted in 200 µL methanol:water (1:1, v/v) before injection into liquid chromatography - mass spectrometry (LC-MS) (Waters Corp, Milford, MA, USA). For quality control purpose, one negative sample and one sample spiked with FB₁ were processed together with each batch of urine samples. The mean concentration of UFB₁ of the two days samples was calculated to represent the exposure. The limit of detection for UFB₁ was 20 pg/ml of urine.

3.8 Statistical Analysis

Statistical analyses were performed using the STATA^(R) 11.1 statistical package (StataCorp LP, Texas USA). A *p*-value of ≤ 0.05 at 95% confidence interval (95% CI) was considered statistically significant. Distributions of concentrations of AF-alb and UFB₁ biomarkers were skewed and therefore were natural logarithmic transformed to normal distribution for statistical analysis. For the purpose of data analysis, samples with AF-alb or UFB₁ levels below limits of detection were assigned half the value of their respective detection limits in accordance with the recommendation of GEMS/Food-EURO (1995).

Differences in means of AF-alb, UFB₁ and growth indices between sampling times and between villages were compared using analysis of variance. Pearson's chi-square test was performed to compare proportions in categorical variables. Correlation between continuous variables was assessed by Pearson's correlation analysis. Multiple regression analyses were used to assess the relationship between levels of mycotoxin exposure biomarkers and child nutritional status or their respective key predictors.

The relationship between aflatoxin or fumonisin exposure and child growth was assessed using; (a) biomarker concentration at recruitment, (b) the mean biomarker concentration of samples collected at recruitment and at 6 months after recruitment and (c) the mean biomarker concentration of samples collected at recruitment, at 6 and at 12 months after recruitment. Separate multivariable regression models were built with each growth indicator treated as the outcome variable and aflatoxin or fumonisin biomarker concentrations as predictor covariates. The models were adjusted for village, breast feeding, maternal education, family SES, protein and energy intakes. Length gain models were additionally adjusted for sex, baseline age in months and baseline length.

Univariate and multivariate binary logistic regression analyses were used to assess the associations between under-nutrition and various variables. In separate models, stunting and underweight were treated as dependent variables with binary outcomes, using $LAZ < -2$ and $WAZ < -2$ as cut-off values respectively. Variables significantly associated with malnutrition in univariate analysis were further investigated in multivariate regression to determine adjusted odds ratio (OR). Risk of being stunted or underweight relative to the predictor variables was determined using OR with 95% CI.

Tracking of dynamic change of nutritional status over time was conducted to determine the extent to which the children from a particular category of nutritional status (such as normal, stunted or underweight) at recruitment maintained their status after one year. The strength of tracking children nutritional status was determined by weighted Kappa values (k).

CHAPTER FOUR

4.0 RESULTS

Results of the study are reported in this chapter. The chapter highlights on child and household demographics and socio-economic data, feeding practices and food intake data, which were gathered through the structured interview. The chapter further describes results of laboratory analysis of AF-alb in plasma samples, analysis of fumonisin B₁ in urine and assessment of nutritional status, presented along with the relevant key variables such as survey periods and study areas. Additionally, the chapter presents results of data analysis to explore the relationships of AF-alb, UFB₁ or nutritional status with the key variables.

4.1 Description of Study Subjects

A total of 166 children, consisting of 78 boys and 88 girls were enrolled for the study. Their mean age at enrolment with 95% CI was 9.7 (9.4 - 10.0) months, ranging from 6 to 14 months. There was no significant difference between villages in terms of children's age at recruitment. The number of subjects per village was 62 in Nyabula (Iringa region), 47 in Kigwa (Tabora region) and 57 in Kikelelwa (Kilimanjaro region), which was obtained based on size of the sampling frame at a village. Distribution of the subjects by age, sex, survey period and village is presented in Table 1. The loss to follow up during the 12 months study was 20 (12%) out of 166, because of migration of 12 (7%) subjects and withdrawal of 8 (5%) subjects.

Table 1: Distribution of subjects by sample size, age and sex

Characteristics	Nyabula village		Kigwa village		Kikelelwa village		All villages	
	n	%	n	%	n	%	n	%
Number of children at recruitment	62	37.3	47	28.3	57	34.3	166	100
Sex:								
Male	31	50	17	36	30	53	78	47
Female	31	50	30	64	27	47	88	53
Age at recruitment (months)								
Mean age (95% CI)	10.0 (9.5 - 10.5)		10.0 (9.6 - 10.4)		9.2 (8.7 - 9.7)		9.7 (9.4 - 10.0)	
Age categories								
6 - 8	20	32.3	10	21.3	27	47.4	57	34.5
9 - 10	31	50.0	32	68.1	25	43.8	88	53.0
12 - 14	11	17.7	5	10.6	5	8.8	21	12.7
Age at 6 months after recruitment (months)								
Mean age (95% CI)	17.1 (16.5 - 17.6)		17.8 (17.3 - 18.2)		16.2 (15.7 - 16.7)		16.9 (16.6 - 17.2)	
Age categories								
12 - 14	19	33.9	5	13.5	25	47.2	49	33.6
15 - 17	27	48.2	28	75.7	25	45.4	80	54.1
18 - 20	10	17.8	4	10.8	5	9.1	19	12.8
Age at 12 months after recruitment (months)								
Mean age (95% CI)	22.6 (22.1 - 23.1)		23.5 (23.1 - 24.0)		21.9 (21.4 - 22.4)		22.5 (22.2 - 22.8)	
Age categories								
18 - 20	19	33.9	5	13.5	25	47.2	49	33.6
21 - 23	27	48.2	28	75.7	23	43.4	78	53.4
24 - 26	10	17.9	4	10.8	5	9.4	19	13.0

4.2 Demographic and Socio-economic Characteristics of Households

Details of demographic characteristics of the children's households at the time of recruitment are presented by village in Table 2. Overall, majority of children (about 96%) were from peasant households. About 89% of mothers had completed primary education. Seventy eight percent of children's mothers were married and 22% were living in female headed households. Average number of births by the mothers was 4 (range 1 to 9). The mean household size was 6 persons, ranging from 2 to 30. In terms of socio-economic status based on procedures prescribed under section 3.6.2, about half (46%) of the subjects were from households of medium SES whereas 43% and 11% were from low and high SES, respectively. By village, Kikelelwa village in Kilimanjaro region had a higher SES compared to Nyabula (Iringa region) and Kigwa (Tabora region) ($p < 0.001$).

Table 2: Household demographic and socio-economic characteristics at recruitment

Characteristic	Nyabula (n = 62)		Kigwa (n = 47)		Kikelelwa (n = 57)		All villages (n = 166)	
	n	%	n	%	n	%	n	%
Children from peasant households	61	98	44	94	54	95	159	96
Mothers' level of education								
Without formal education	2	3	10	21	1	2	13	8
Primary	59	95	36	77	52	91	147	89
Secondary	1	2	1	2	4	7	6	3
Maternal marital status								
Mothers who were married	45	73	41	87	43	75	129	78
Living single	17	27	6	13	14	25	37	22
Family SES categories								
Low	39	63	32	68	0	0	71	43
Medium	22	35	11	23	43	75	76	46
High	1	2	4	9	14	25	19	11
Mothers' number of births								
Mean (95% CI)	3.8 (3.2 - 4.4)		4.3 (3.5 - 5.1)		3.3 (2.8 - 3.8)		3.8 (3.4 - 4.1)	
Range	1 - 9		1 - 9		1 - 7		1 - 9	
Family size								
Mean (95% CI)	5.4 (4.9 - 5.9)		8.2 (6.5 - 9.8)		6.0 (5.2 - 6.7)		6.3 (5.8 - 6.9)	
Range	2 - 11		3 - 30		3 - 19		2 - 30	

4.3 Child Birth Weight and Immunisation

Data on children's birth weights data was available for only 106 (64%) of the recruited children who were delivered at hospitals, dispensaries or health centres. Birth weight data was missing in 36% of children who were delivered at home. Among the children whose birth weight data were available, 92% were born with normal weight (2.5 kg and above) and 8% had low birth weight (less than 2.5 kg). The proportion of children born with low birth weight was 15%, 9% and 0% at Nyabula, Kigwa and Kikelelwa respectively. The overall mean birth weight (with 95% CI) was 3.1 (3.0 - 3.2) kg. Record from health clinic card showed that 90.4% of the studied children were immunised against vaccine preventable diseases (tuberculosis, polio, diphtheria, pertussis, tetanus, hepatitis B and

measles) and had received at least the first dose of vitamin A supplement (100 000 IU at nine months of age).

4.4 Age at Complementary Feeding and Children's Breastfed Status

The overall mean age at which the children were first introduced into complementary foods was 4 months, ranging from 0 - 6 months. About three quarters (76%) of the children were introduced to complementary foods before attaining the age of 6 months and 8% started complementary feeding even before they were 2 months old. At recruitment, none of the children was on exclusive breast feeding. Ninety three percent of the children (at 6 to 14 months of age) were partially breastfed (breastfed but also given complementary foods). Due to increased reliance on complementary foods as children grew older, the proportion of breastfed children decreased from 93% at recruitment to 78% and 34% at 6 months and at 12 months after recruitment respectively. There was no association between child breastfed status and household SES. Details of age at introduction of complementary foods and children breastfed status by village are presented in Table 3.

Table 3: Age of complementary feeding and breastfed status

Variable	Nyabula	Kigwa	Kikelelwa	All villages
Age of complementary feeding, mean (95% CI) (months)	4.5 (4.1 - 4.9)	3.7 (3.2 - 4.2)	3.4 (3.0 - 3.7)	3.9 (3.6 - 4.2)
Specific age at complementary feeding (months), %				
< 2	3	15	7	8
2 to < 4	23	23	53	33
4 to < 6	31	45	31	35
6	43	17	9	24
Breastfed (i.e partially breastfed*), %				
At recruitment	90	100	91	93
6 months after recruitment	77	73	82	78
12 months after recruitment	38	8	49	34

* Breastfed and given complementary foods

4.5 Complementary Foods

Complementary foods for the children were composed mainly of ingredients from locally available food products. Common foods were maize porridges (thin porridge and stiff porridge), bananas, potatoes, rice, finger millet, beans, cassava, groundnuts, meat, fresh cow's milk, eggs, vegetables and fruits. However, consumption of some of these foods differed by village depending on the dominant food crops grown in the area. Consumption of maize and maize-based products was more dominant among children in Nyabula village (Iringa region) where maize was the dominant crop from subsistence farming. Maize and rice consumption was more common in children in Kigwa village (Tabora region) and at Kikelelwa village (Kilimanjaro region), the dominant food ingredients were bananas, potatoes and maize. The estimated mean protein intake by child per day at the time of recruitment was 9.4 ± 3.5 g and estimated mean energy intake was 644 ± 220 kcal. These intakes increased to 13 ± 4.4 g (protein) and 710 ± 213 kcal (energy) at 6 months after recruitment and to 17 ± 2.2 g (protein) and 834 ± 189 kcal (energy) at 12 months after recruitment. This increase is attributed to increased amount of food intake associated with increased body requirements as children grew older.

4.6 Estimated Quantities of Maize Intake by Children

Amounts of maize intake were determined as described under section 3.6.3.2. Maize was reported to be consumed by all studied children. The overall mean (with 95% CI) intake of maize by child was 8.8 (8.1 - 9.5), 9.8 (8.9 - 10.6) and 12.0 (11.1 - 12.9) g/kg bw/day at recruitment, at 6 months and at 12 months after recruitment, respectively. These levels were significantly higher at 12 months after recruitment than during the previous surveys ($p < 0.001$). The mean maize intake at recruitment presented by village was significantly higher at Nyabula than Kigwa and Kikelelwa ($p < 0.01$). At 6 months after recruitment, the intake was significantly higher at Nyabula village than Kigwa village ($p < 0.001$) and

Kikelelwa village ($p < 0.01$) and at 12 months after recruitment the intake was significantly lower in children at Kikelelwa village than those at Nyabula village ($p < 0.01$) and Kigwa village ($p < 0.05$) (Table 4). There was no significant difference in maize intake between boys and girls. Overall, the number of days per week (frequency) at which children was fed on maize based complementary foods ranged from 1 to 7. Up to 98% of children at Nyabula and Kigwa villages and 61% at Kikelelwa were reported to be on maize based food in seven days per week.

Table 4: Estimated quantities of maize consumption by children

Variable	Amount of maize intake [mean (95% CI) (g/kg bw/day)]			
	Nyabula	Kigwa	Kikelelwa	All villages [*]
At recruitment	10.6 (9.5 - 11.7) ^a	7.8 (6.6 - 9.0) ^b	7.7 (6.9 - 8.8) ^b	8.8 (8.1 - 9.5)
6 months after recruitment	12.1 (10.6 - 13.7) ^a	8.9 (6.9 - 10.8) ^b	7.9 (6.9 - 8.8) ^b	9.8 (8.9 - 10.6)
12 months after recruitment	13.4 (12.2 - 14.6) ^a	12.9 (11.3 - 14.5) ^a	9.9 (8.3 - 11.5) ^b	12.0 (11.1 - 12.9)

*Significantly higher maize intake at 12 months after recruitment than at the previous surveys ($p < 0.001$)

^{a, b} Significant difference between villages in terms of mean intakes marked with different letters using ANOVA

4.7 Maize Intake by Breastfed Status

Distribution of quantities of maize intake by child breastfed status is illustrated in Fig. 6. Quantity of maize intake was found to be higher in non-breastfed children than in the children who were breastfed.

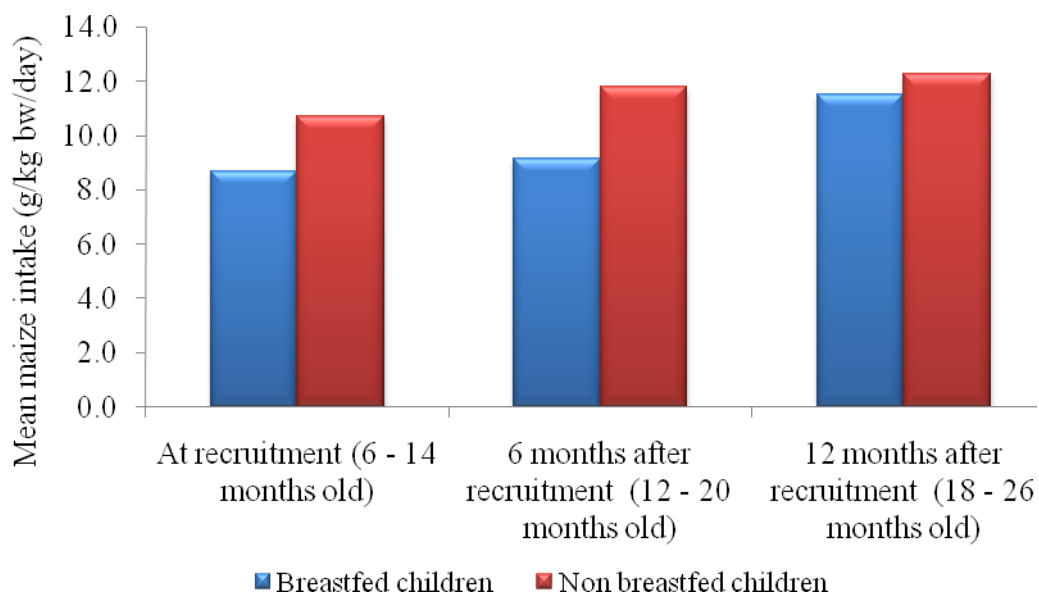


Figure 6: Quantities of maize intake by children's breastfed status

4.8 Consumption of Groundnuts by Children

It was reported that groundnuts were consumed in different forms such as mixed with maize and other cereals to make composite flour, ground into paste/flour and added into family dishes of vegetable based sauce or eaten as snacks. In view of this, reliable estimation of quantities of groundnuts consumption was difficult, thus only information on frequency of consumption was obtained. The percentage of children reported to consume groundnuts during the previous week before each survey and number of days per week during which groundnuts were consumed is summarised in Fig. 7 and Table 5, respectively. About 19%, 12% and 23% of the children at Nyabula village were reported to consume groundnuts at recruitment, at 6 and at 12 months after recruitment respectively. At these respective survey periods, 32%, 32% and 78% of children at Kigwa village and 21%, 15% and 25% at Kikelelwa village were also reported to consume groundnuts (Fig. 7). More children were reported to consume groundnuts in Kigwa village compared to Nyabula and Kikelelwa villages.

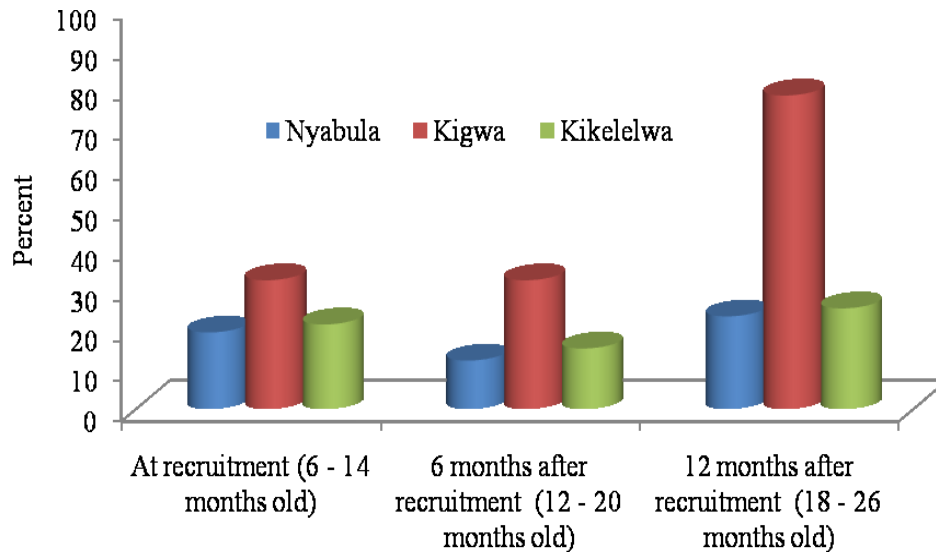


Figure 7: Percentage of children consuming groundnuts by village

Table 5: The percentage of children who consumed groundnuts by number of days per week

Survey	Village	Number of days per week		
		0	1 to 3	4 to 7
At recruitment	Nyabula	81	13	6
	Kigwa	68	2	30
	Kikelelwa	79	5	16
6 months after recruitment	Nyabula	87	4	9
	Kigwa	68	24	8
	Kikelelwa	85	7.5	7.5
12 months after recruitment	Nyabula	77	11	12
	Kigwa	22	65	13
	Kikelelwa	75	17	8

4.9 Dietary Exposure of Children to Aflatoxin

4.9.1 Aflatoxin exposure at recruitment to the study

Plasma samples were obtained from 147 (89%) children out of the recruited 166 children. About 67% of them were detected with AF-alb biomarker (Table 6). The prevalence of children who were detected with AF-alb was 40% at Nyabula, 95% at Kigwa and 73% at

Kikelelwa villages, respectively. The prevalence varied significantly between the villages ($p < 0.001$). Concentrations of AF-alb are summarised in Table 7 and illustrated in Fig. 8. The overall geometric mean concentration of AF-alb at 95% CI was 4.7 (3.9 - 5.6) pg/mg. Children at Kigwa village demonstrated significantly higher mean concentration at 9.3 (7.0 - 11.6) pg/mg, compared to 3.0 (2.1 - 4.1) pg/mg at Nyabula ($p < 0.001$) and 4.6 (3.4 - 6.1) pg/mg at Kikelelwa ($p < 0.01$).

4.9.2 Aflatoxin exposure at 6 months after recruitment

Plasma samples were obtained from 146 children out of 148 who were available for follow-up at 6 months after recruitment. Of the 146 children, plasma samples from 84% of them tested positive for AF-alb (Table 6). The prevalence was 96% at Nyabula (Iringa region), 97% at Kigwa (Tabora region) and 61% at Kikelelwa (Kilimanjaro region). Prevalence of AF-alb positive children was significantly lower at Kikelelwa than other villages ($p < 0.001$). The overall geometric mean concentration of AF-alb (95% CI) was 12.9 (9.9 - 16.7) pg/mg albumin. Children at Kigwa village had the highest AF-alb mean concentration at 43.2 (28.7-65.0) pg/mg, followed by Nyabula at 19.9 (13.5-29.2) pg/mg and Kikelelwa had the lowest concentration at 3.6 (2.8 - 4.7) pg/mg. The mean AF-alb concentration varied significantly between the villages ($p < 0.001$) (Table 7 and Fig. 8).

4.9.3 Aflatoxin exposure at 12 months after recruitment

Plasma samples were obtained from 143 children out of 146 who were available during the final survey (i.e 12 months after recruitment). Of the 143 children, plasma samples from all but one child from Kikelelwa village had detectable levels of AF-alb (Table 6). The overall geometric mean concentration (95% CI) was 23.5 (19.9 - 27.7) pg/mg albumin. The levels by village were 20.8 (16.2 - 26.1) pg/mg, 48.8 (34.5 - 69.1) pg/mg and 16.1 (12.6 - 20.7) pg/mg at Nyabula, Kigwa and Kikelelwa respectively. Children from Kikelelwa village

demonstrated the lowest mean concentration of AF-alb compared to Nyabula and Kigwa ($p < 0.001$) (Table 7 and Fig. 8).

Table 6: Prevalence of children detected with AF-alb by village and survey period

Survey	Nyabula ^a	Kigwa ^b	Kikelelwa ^c	Overall	<i>p</i> -value
	(%)	(%)	(%)	(%)	
At recruitment	40*	95**	73***	67	<0.001
6 months after recruitment	96*	97*	61**	84	<0.001
12 months after recruitment	98	100	100	99	0.436

^aAt Nyabula village, prevalence was significantly lower at recruitment than during the other two surveys ($p < 0.001$)

^bAt Kigwa village, there was no significant difference in prevalence between the three surveys ($p = 0.410$)

^cAt Kikelelwa village, prevalence was significantly higher at 12 months after recruitment than during the preceding surveys ($p < 0.001$)

*, **, *** Significant difference in prevalence between groups (villages) marked with different asterisks within a survey

Table 7: Distribution of AF-alb concentrations by survey period and village (pg/mg albumin)

Village	At recruitment*		6 months After recruitment**		12 months After recruitment***		<i>p</i> -value ^d
	Mean	95% CI	Mean	95% CI	Mean	95% CI	
Nyabula	3.0 ^a	2.1 - 4.1	19.9 ^b	13.5 - 29.2	20.8 ^b	16.2 - 26.1	< 0.001
Kigwa	9.3 ^a	7.0 - 11.6	43.2 ^b	28.7 - 65.0	48.8 ^b	34.5 - 69.1	0.011
Kikelelwa	4.6 ^a	3.4 - 6.1	3.6 ^a	2.8 - 4.7	16.1 ^b	12.6 - 20.7	< 0.001
Overall	4.7 ^a	3.9 - 5.6	12.9 ^b	9.9 - 16.7	23.5 ^c	19.9 - 27.7	< 0.001

* Significantly higher levels in children at Kigwa than Nyabula ($p < 0.001$) and Kikelelwa ($p < 0.01$) at recruitment.

** Significant difference in exposure levels between each village at 6 months after recruitment.

*** Significantly lower levels in children at Kikelelwa than other villages ($p < 0.001$) at 12 months after recruitment.

^{a, b, c} Significant difference between means marked with different letters between surveys within a village. The overall levels differed significantly between surveys ($p < 0.001$).

^d *p*-value for comparing concentrations among survey periods in the specified village.

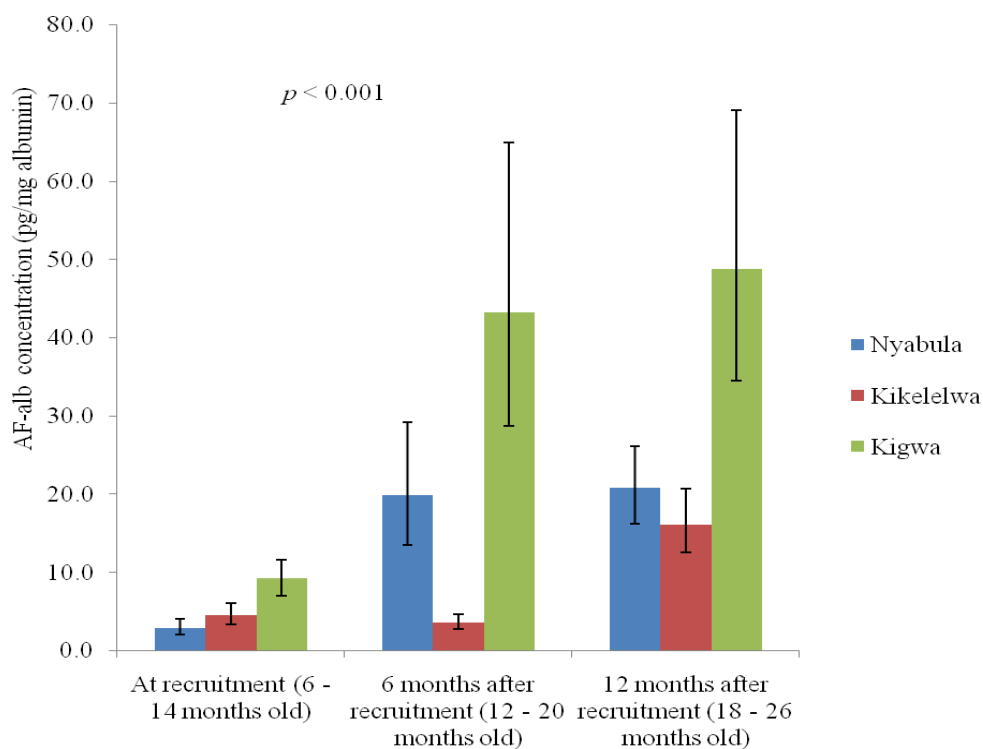


Figure 8: Distribution of AF-alb levels by village at survey periods

4.9.4 Distribution and trend of aflatoxin exposure by survey period, village and sex

Overall, there was a consecutive significant increased trend of both the prevalence of children whose plasma samples tested positive for AF-alb and in mean levels of AF-alb between the three surveys ($p < 0.001$) (Fig. 9). Consistently, children at Kigwa village (Tabora region) showed the highest levels of AF-alb across the three surveys, followed by Nyabula and Kikelelwa (Table 7). When mean AF-alb levels from all three survey periods were combined for each village, again children at Kigwa village had the highest exposure, followed by Nyabula and Kikelelwa (Fig. 10). The mean exposure levels from the three surveys differed significantly between the villages ($p < 0.001$). At each village, there was a negative but non-significant correlation between mean AF-alb levels from the three surveys for each child and SES scores. A multivariate linear regression analysis was conducted to determine the key predictors for aflatoxin exposure, where by quantities of maize

consumption, SES, child's age and village of residence were included in the model. The results showed that at each survey, village was a key determinant of aflatoxin exposure (Table 8). During each survey, there was no substantial difference by sex in levels of AF-alb.

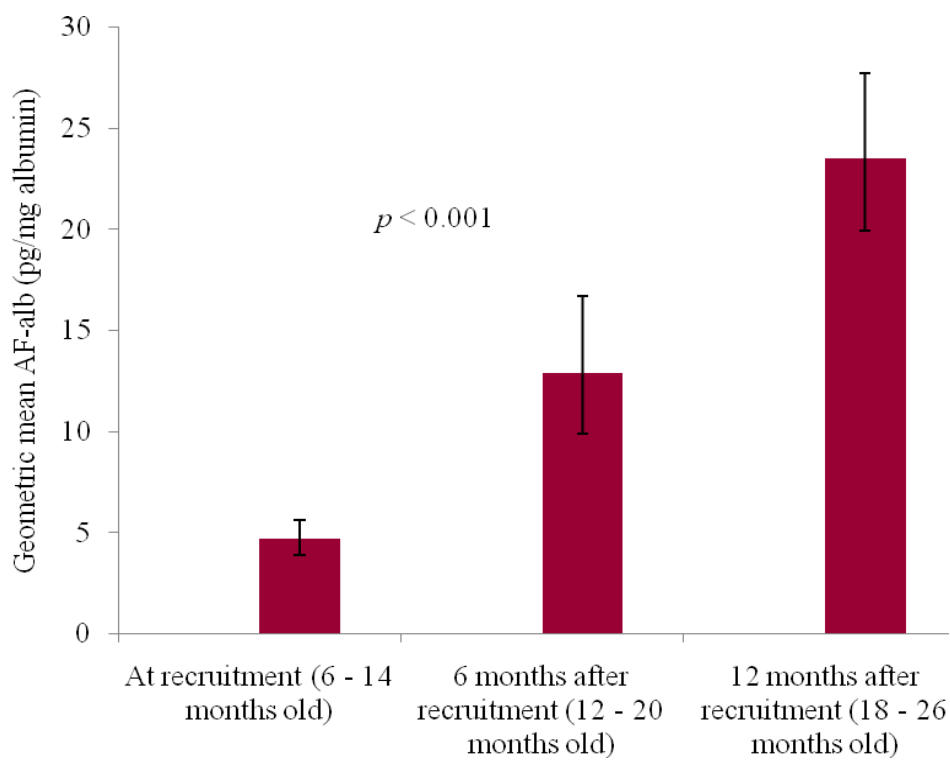


Figure 9: Distribution of AF-alb levels by survey periods

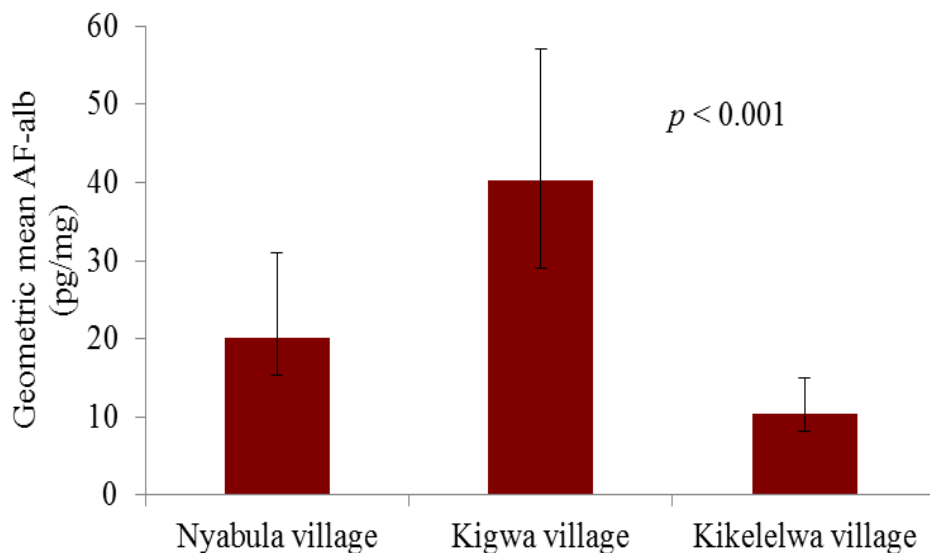


Figure 10: Distribution of mean AF-alb levels from all survey periods by village

Table 8: Multiple regression analysis for the determinants of aflatoxin exposure

Predictor variable	At recruitment		6 months after recruitment		12 months after recruitment	
	β	<i>p</i> value	β	<i>p</i> value	β	<i>p</i> value
Maize intake (g/kg bw/day)	-0.019	0.370	0.049	0.012*	0.005	0.751
SES (Low versus medium)	-0.024	0.918	-0.406	0.120	0.062	0.763
SES (Low versus high)	0.055	0.875	-0.662	0.074	-0.185	0.516
Age (months)	0.059	0.239	0.122	0.026*	0.071	0.094
Nyabula versus Kikelelwa village	0.408	0.131	-1.063	0.000*	-0.166	0.466
Nyabula versus Kigwa village	1.037	0.000*	0.890	0.001*	0.805	0.000*

β = Regression coefficient

*Significant level

4.9.5 Correlation of AF-alb levels with child age and quantities of maize intake

There was a positive correlation between levels of AF-alb and quantities of maize intake per child at 6 months and at 12 months after recruitment. This correlation was significant at 6 months after recruitment ($r = 0.267$; $p = 0.001$) (Table 9) and remained significant even after adjustment for SES, child's age and village in multiple regression analysis (Table 8). During each survey, levels of AF-alb correlated positively with child's age and this was

significant at 6 months ($r = 0.326$; $p < 0.001$) and at 12 months after recruitment ($r = 0.266$; $p = 0.0013$) (Table 9).

Table 9: Correlation of AF-alb levels with quantities of maize intake and child age

Survey	Quantity of maize intake		Child age	
	r	p value	r	p value
At recruitment	-0.148	0.074	0.096	0.2460
6 months after recruitment	0.267	0.011*	0.326	0.0001*
12 months after recruitment	0.076	0.366	0.266	0.0013*

r = correlation coefficient

*Significant level

4.9.6 AF-alb levels correlated with groundnuts consumption

At each survey, the children who were reported to consume groundnuts demonstrated higher concentrations of AF-alb than those who did not consume groundnuts; 7.5 (4.7 - 12.1) pg/mg versus 4.0 (3.3 - 4.9) pg/mg at recruitment ($p < 0.01$) and 22.8 (12.3- 42.4) pg/mg versus 11.3 (8.5 - 15.0) pg/mg at 6 months after recruitment ($p < 0.05$) (Fig. 11). At 12 months after recruitment, the levels were also high in children who consumed groundnuts compared to those who did not consume groundnuts; 28.1 (21.3 - 37.1) pg/mg versus 21.0 (17.1 - 25.7) pg/mg although the difference was not significant. There was significant positive correlation between mean levels of AF-alb from the three surveys and frequency of consumption of groundnuts per week ($r = 0.22$; $p < 0.01$).

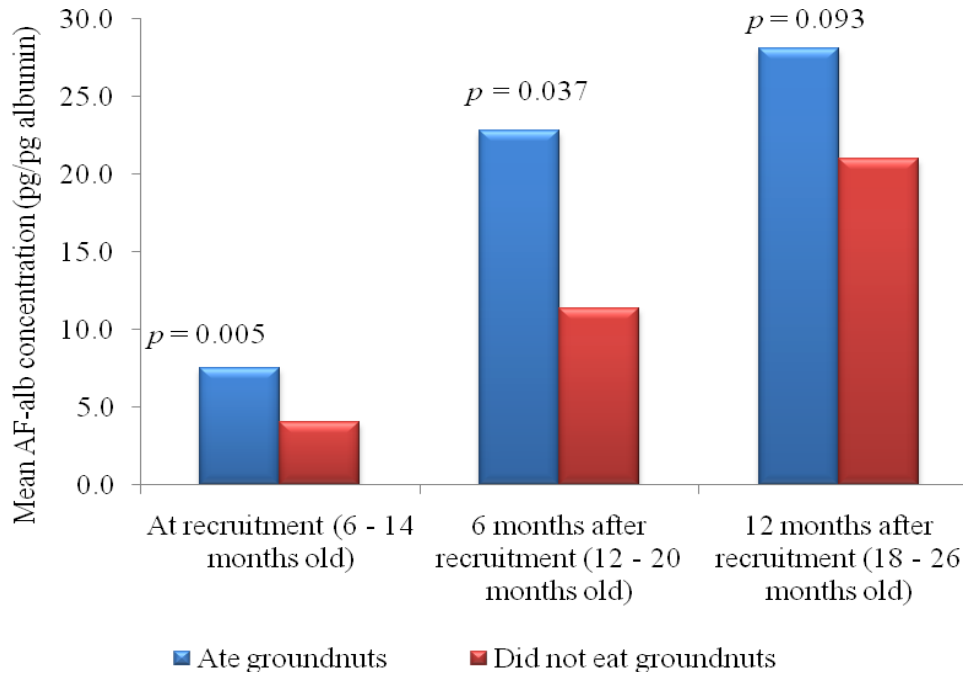


Figure 11: Mean AF-alb concentrations in children compared to groundnuts consumption status

4.9.7 Association of AF-alb with breastfed status

Non-breastfed children demonstrated high levels of AF-alb compared to the children who in addition to eating complementary feeding were also breastfed. At recruitment, the children who were breastfed had slightly lower mean concentration of AF-alb than the non-breastfed children [4.63 (3.83 - 5.59) pg/mg versus 5.37 (1.93 - 14.95) pg/mg]. During the second survey (6 months after recruitment when children were 12 -20 months old), the mean level in non-breastfed children was more than double that in breastfed children [24.7 (14.32 - 42.59) pg/mg versus 10.73 (8.03 - 14.34 pg/mg)] ($p = 0.008$). A similar trend was observed at 12 months after recruitment whereby non-breastfed children were found with higher levels of AF-alb at 28.1 (22.8 - 34.5) pg/mg compared to 16.6 (12.9 - 21.48) pg/mg in the breastfed children ($p = 0.0028$) (Fig. 12).

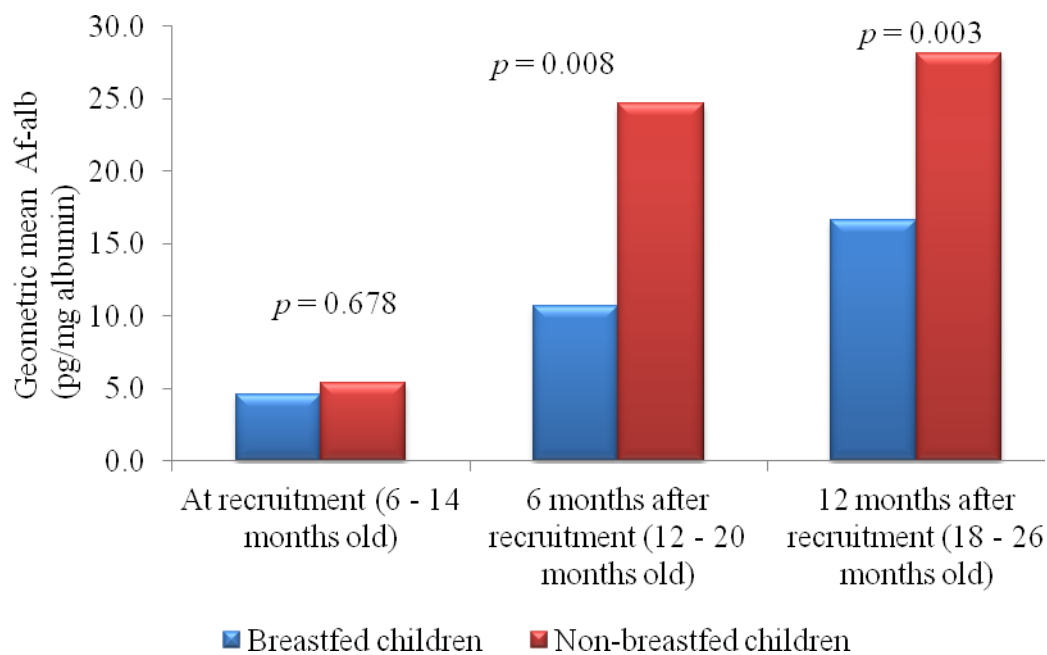


Figure 12: Comparison of AF-alb levels by child breastfed status

4.10 Dietary Exposure of Children to Fumonisin

4.10.1 Fumonisin exposure at recruitment to the study

Urine samples were obtained from 160 (96%) children out of 166 who were recruited and 98% were detected with fumonisin B₁ in their urine (Table 10). All the children at Nyabula and Kigwa and 94% at Kikelelwa village were exposed to fumonisins. The overall geometric mean level of UFB₁ with 95% CI was 313.9 (257.4 - 382.9) pg/ml (Table 11). Children at Kigwa village had significantly higher levels of UFB₁ at 544.2 (397.2 - 745.6) pg/ml than children at Kikelelwa village at 199.7 (137.7 - 289.6) pg/ml ($p < 0.001$). The mean exposure levels at Nyabula village was 312.2 (230.0 - 424.1) pg/ml (Table 11 and Fig. 13).

4.10.2 Fumonisin exposure at 6 months after recruitment

A total of 148 children were available for follow-up at sixth month after recruitment and out of them, 147 had urine samples collected for analysis. About 96% of the collected urine

samples had positive UFB₁ levels (Table 10). The overall geometric mean level of UFB₁ (95% CI) was 167.3 (135.4 - 206.7) pg/ml of urine (Table 11 and Fig. 13). Children at Kigwa village demonstrated the highest mean level of UFB₁ at 327.2 (217.1 - 493.0) pg/ml, followed by Nyabula at 211.7 (161.1 - 278.1) pg/ml then Kikelelwa at 82.8 (58.3 - 117.7) pg/ml. The difference was statistically significant between Kikelelwa and the other two villages ($p < 0.001$).

4.10.3 Fumonisin exposure at 12 months after recruitment

At 12 months after recruitment, urine samples were collected from all 146 children who were available for the final survey. All the urine samples tested positive for UFB₁ (Table 10). The overall geometric mean level of UFB₁ (95% CI) was 569.5 (464.5 - 698.2) pg/ml of urine. Children at Kikelelwa village had the lowest levels of UFB₁ at 320.2 (228.9 - 448.1) pg/ml compared to 868.3 (617.9 - 1220.0) pg/ml at Nyabula ($p < 0.001$) and 686.1 (505.4 - 931.5) pg/ml at Kigwa ($p < 0.01$) (Table 11 and Fig. 13).

Table 10: Prevalence of children detected with urinary fumonisin B₁ by village and survey period

Survey	Nyabula (%)	Kigwa (%)	Kikelelwa ^a (%)	Overall (%)	<i>p</i> -value
At recruitment	100	100	95	98	0.054
6 months after recruitment	100*	100*	89**	96	0.003
12 months after recruitment	100	100	100	100	

^aAt Kikelelwa village, the prevalence was significantly lower at 6 months after recruitment than during the other two surveys ($p < 0.05$)

*, ** At 6 months after recruitment, there was lower prevalence at Kikelelwa village compared to Nyabula and Kigwa ($p < 0.01$)

Table 11: Distribution of UFB₁ concentrations by survey period and village (pg/ml urine)

Village	At recruitment*		6 months after recruitment**		12 months After recruitment***		<i>p</i> – value ^d
	Mean	95% CI	Mean	95% CI	Mean	95% CI	
Nyabula	312.2 ^a	230.0 - 424.1	211.7 ^a	161.1 - 278.1	868.3 ^b	617.9 - 1220.0	<i>p</i> < 0.001
Kigwa	544.2	397.2 - 745.6	327.2 ^a	217.1 - 493.0	686.1 ^b	505.4 - 931.5	<i>p</i> < 0.001
Kikelelwa	199.7 ^a	137.7 - 289.6	82.8 ^b	58.3 - 117.7	320.2 ^a	228.9 - 448.1	<i>p</i> < 0.01
Overall	313.9 ^a	257.4 - 382.9	167.3 ^b	135.4 - 206.7	569.5 ^c	464.5 - 698.2	<i>p</i> < 0.001

*Significantly higher levels of UFB₁ in children at Kigwa than Kikelelwa (*p* < 0.001) during recruitment

** Significantly lower levels of UFB₁ in children at Kikelelwa village than other villages (*p* < 0.001) at 6 months after recruitment

*** Significantly lower levels of UFB₁ in children at Kikelelwa village than Nyabula (*p* < 0.001) and Kigwa (*p* < 0.01) at 12 months after recruitment

^{a, b, c} Significant difference between means marked with different letters between surveys within a village. The overall levels were higher at 12 months after recruitment than during the previous surveys (*p* < 0.001)

^d *p*-value for comparing concentrations among survey periods in the specified village

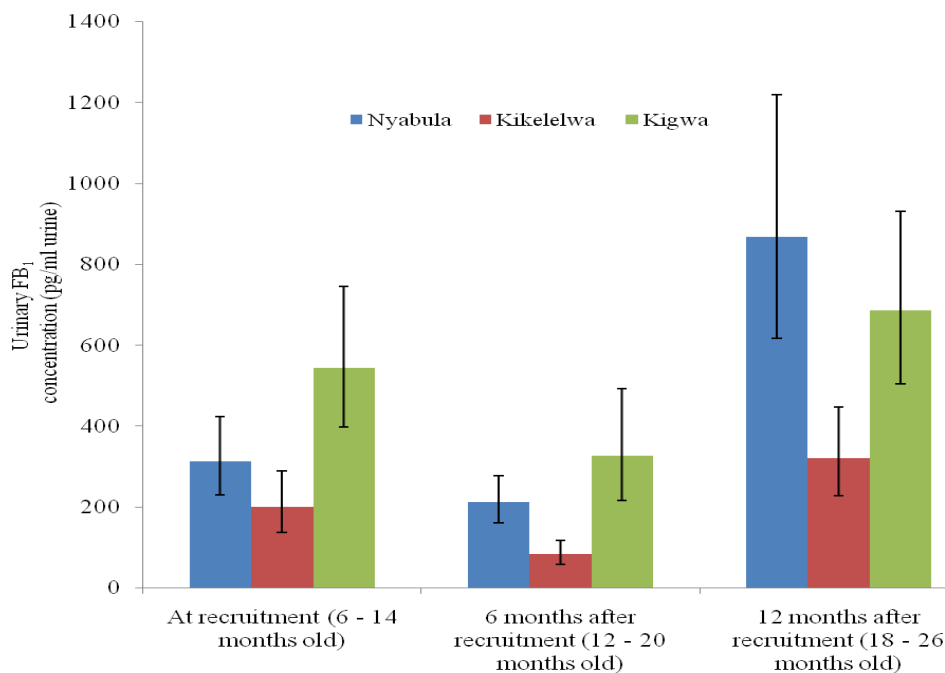


Figure 13: Distribution of UFB₁ levels by village at survey periods

4.10.4 Distribution and trend of fumonisin exposure in children by survey periods

There were significantly higher levels of UFB₁ at 12 months after recruitment than during the preceding surveys ($p < 0.001$). The UFB₁ mean level at 6 months after recruitment when the children were 12 - 20 months old was lower than at recruitment when they were 6 - 14 months old ($p < 0.001$) (Fig. 14).

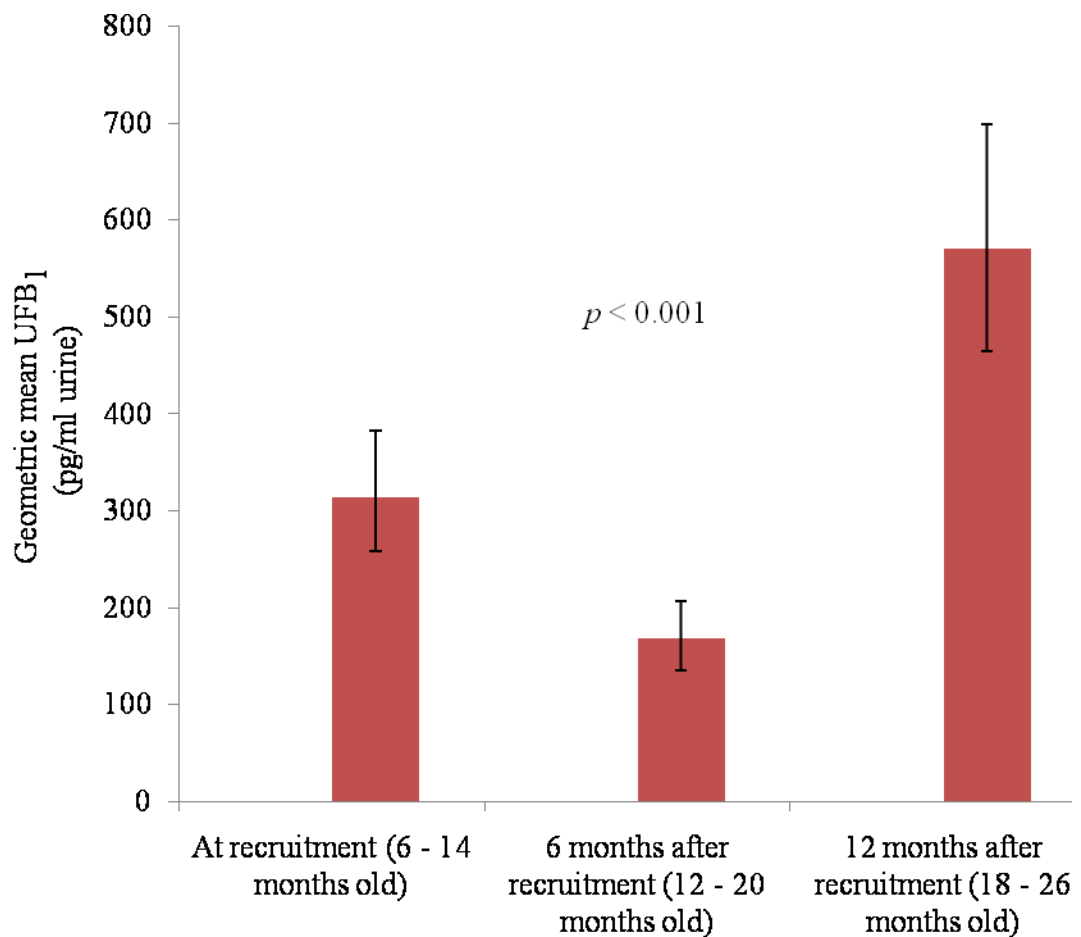


Figure 14: Distribution of UFB₁ levels by survey period

4.10.5 Distribution and trend of fumonisin exposure in children by village

When the mean UFB₁ levels from all the three surveys was calculated per village (Fig. 15), children from Kikelelwa village demonstrated the lowest levels at 156.6 (117.8 - 208.3) pg/ml. Levels in children from the other villages were 307.8 (244.0 - 388.2) pg/ml at Nyabula and 470.5 (334.7 - 661.2) pg/ml at Kigwa village. The exposure levels among

children at Kikelelwa village were significantly lower than those at Nyabula village ($p < 0.01$) and Kigwa village ($p < 0.001$) (Fig. 15). At each village, there was a negative but non-significant correlation between SES scores and mean UFB₁ levels from the three surveys for each child. Furthermore, multivariate regression analysis demonstrated village of residence as a significant determinant of magnitude of exposure to fumonisin at each survey (Table 12). There was no significant difference in the mean UFB₁ levels between boys and girls.

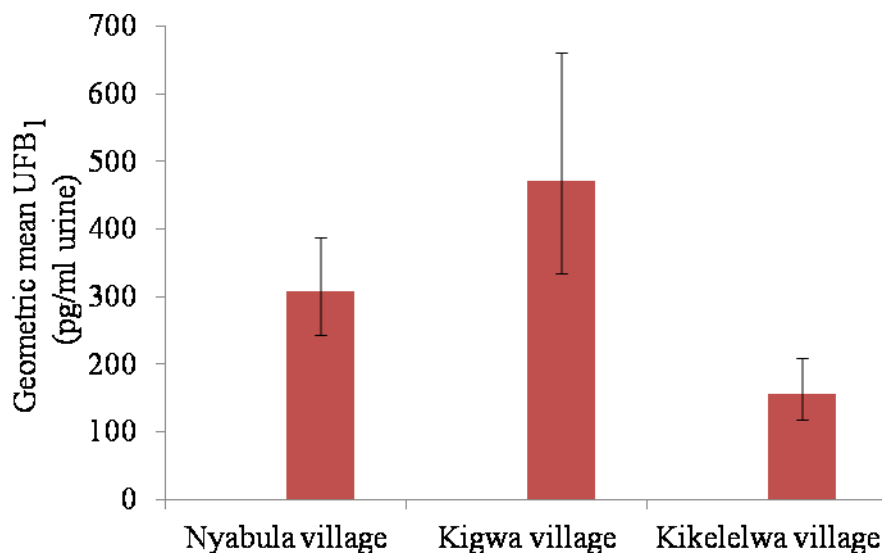


Figure 15: Distribution of mean UFB₁ concentration from all survey periods by village

Table 12: Multiple regression analysis for the determinants of fumonisin exposure

Predictor variable	Recruitment		6 months after recruitment		12 months after recruitment	
	β	<i>p</i> value	β	<i>p</i> value	β	<i>p</i> value
Maize intake (g/kg bw/day)	0.078	0.001*	0.043	0.027*	0.051	0.011*
SES (Low versus medium)	-0.183	0.456	-0.133	0.607	-0.084	0.746
SES (Low versus high)	-0.259	0.470	-0.229	0.534	-0.465	0.200
Age (months)	-0.008	0.882	-0.027	0.612	0.041	0.445
Nyabula versus Kikelelwa village	-0.107	0.710	-0.668	0.026*	-0.643	0.028*
Nyabula versus Kigwa village	0.723	0.003*	0.610	0.020*	-0.212	0.401

β = Regression coefficient

*Significant level

4.10.6 Correlation between levels of UFB₁ and quantities of maize intake

There was significant positive correlation between UFB₁ levels and maize intake by children at recruitment ($r = 0.261$; $p < 0.001$), at 6 months after recruitment ($r = 0.241$; $p < 0.01$) and at 12 months after recruitment ($r = 0.288$; $p < 0.001$). Higher UFB₁ levels were associated with higher maize intake at each survey period after adjustment for SES, child's age, and village in multivariate regression model (Table 12). In the multivariate model, the association at each survey was as follows; at recruitment ($\beta = 0.078$; $p < 0.01$), at 6 months after recruitment ($\beta = 0.043$; $p < 0.05$) and at 12 months after recruitment ($\beta = 0.051$; $p < 0.05$).

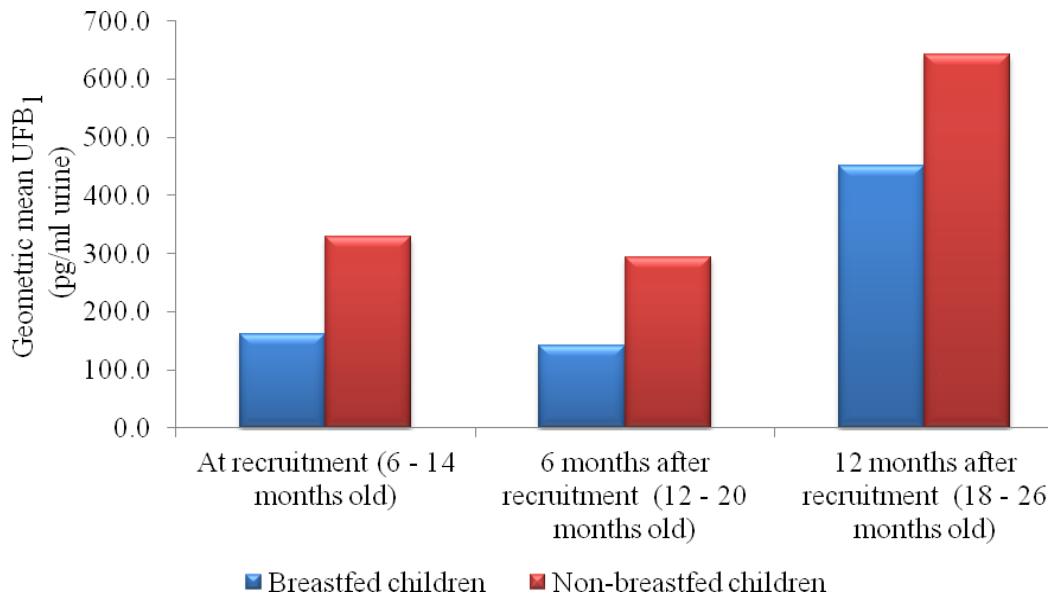
4.10.7 UFB₁ Levels associated with breastfed status

Mean levels of UFB₁ were higher in children who were eating complementary foods without breastfed than the children who were eating complementary foods in addition to breastfed. This trend was exhibited at each survey period (Table 13 and Fig. 16).

Table 13: Breastfed status and mean UFB₁ levels

Survey period	Breastfed [pg/ml urine (95% CI)]	Non-breastfed [pg/ml urine (95% CI)]	<i>p</i> value
At recruitment	161.3 (57.5 - 452.8)	329.7 (269.7 - 403.1)	0.072
6 months after recruitment	142.2 (112.2 - 180.2)	293.2 (189.4 - 453.7)	0.004*
12 months after recruitment	451.7 (337.3 - 604.9)	642.5 (490.3 - 842.0)	0.105

*Significant difference in UFB₁ concentration between breastfed and the non-breastfed children

**Figure 16: Distribution of UFB₁ levels by breastfed status**

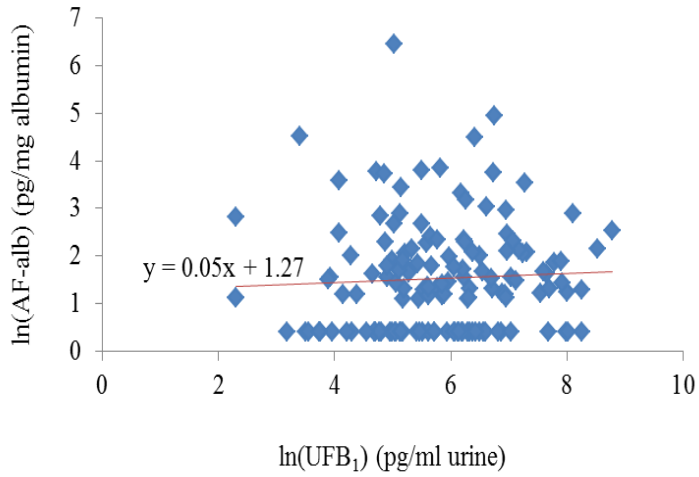
4.11 Relationship Between Mycotoxin Exposure and Maize Processing Methods

During the three survey periods, up to 96% of children's households at Nyabula village (Iringa region), 84% at Kigwa village (Tabora region) and less than 10% at Kikelelwa village (Kilimanjaro region) reported to dehull the maize before it is milled into flour. In addition to dehulling, some households (about 7%) at Nyabula village reported to soak the dehulled maize prior to milling.

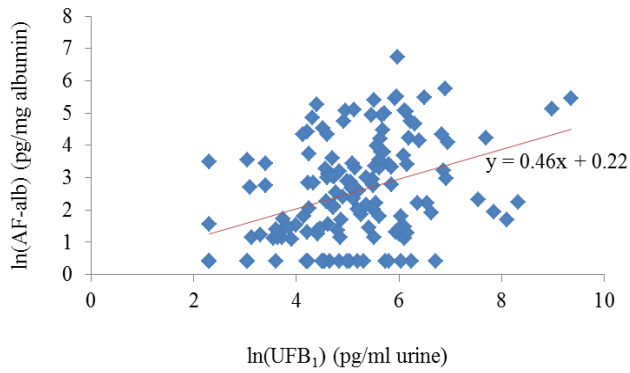
Since the majority of the children at Nyabula village (up to 96%) and Kikelelwa villages (up to 90%) consumed dehulled and whole kernel maize, respectively throughout the three surveys, it was therefore impractical to compare exposure levels in relation to maize processing within each of or between these two villages. However, statistical comparison of levels of mycotoxin exposure and maize dehulling status was only done for Kigwa village at 6 months after recruitment where 49% of children were reported to consume dehulled maize and 51% consumed whole kernel maize. At this village, children who were reported to use whole kernel maize in their complementary foods were detected with significantly higher mean concentration of UFB₁ than the children who consumed dehulled maize [492.3 (255.1 - 949.9) pg/ml versus 212.5 (134.3 - 336.4) pg/ml] ($p = 0.0360$). The mean levels of AF-alb were also higher in children who consumed whole kernel maize [56.2 (34.5 - 91.5 pg/mg)] than in children who consumed dehulled maize [32.8 (16.4 - 65.4)] although the difference were not statistically significance ($p > 0.05$).

4.12 Co-exposure and Correlation of AF-alb and UFB₁

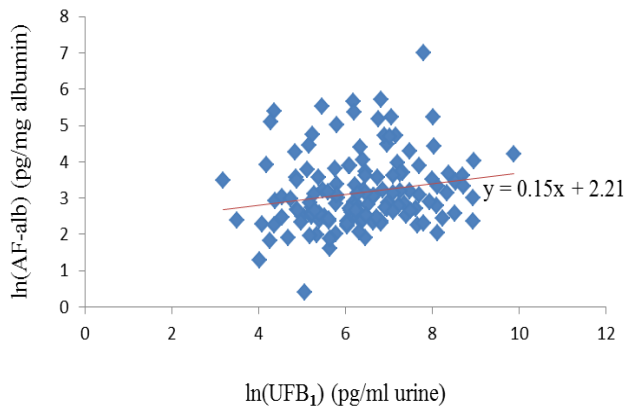
Co-exposure was observed in 64%, 82% and 99% of the screened children who had both AF-alb and UFB₁ at recruitment, at 6 months and at 12 months after recruitment, respectively. During each survey period, there was a positive correlation between levels of AF-alb and UFB₁. This correlation was significant at 6 months ($r = 0.375$; $p < 0.001$) and at 12 months after recruitment ($r = 0.19$; $p < 0.05$) (Fig. 17).



(a) At recruitment (6 - 14 months old)



(b) 6 months after recruitment (12 - 20 months old)



(c) 12 months after recruitment (18 - 26 months old)

Figure 17: Scatter plots of AF-alb and UFB₁ levels at each survey period

4.13 Child Nutritional Status

4.13.1 Prevalence of stunting

Stunting was the most prevalent form of malnutrition throughout the study. At recruitment when children were aged between 6 to 14 months, almost half of them (44%) were stunted, of which 16.3% were severely stunted. At 6 months after recruitment, the prevalence of stunting increased to 55% of which 17.6% were severely stunted. At 12 months after recruitment, the prevalence of stunting further increased to 56% of which 18.5% were severely stunted (Table 14). Overall, prevalence of stunting was significantly lower at recruitment than at 12 months later ($p < 0.05$). When children were categorised by their age at recruitment to the study, those who were 6 to 8 months (youngest group) demonstrated the lower prevalence of stunting (40%) at recruitment and the higher prevalence (71%) at 12 months after recruitment compared to the children who were 9 - 11 or 12 - 14 months old at recruitment (Fig. 18).

At each survey period, there was village variation in the magnitude of stunting. At recruitment, the prevalence was 21.3% in children at Kigwa compared to 55% at Nyabula and 51% at Kikelelwa ($p < 0.01$). At 6 months after recruitment, the prevalence was 71% at Nyabula, 57% at Kigwa and 38% at Kikelelwa. At 12 months after recruitment, the prevalence was 77% at Nyabula, 42% at Kikelelwa and 46% at Kigwa ($p < 0.001$). Likewise, there were village differences in terms of LAZ scores, again with children at Nyabula village demonstrating the lowest scores throughout the surveys ($p < 0.001$) (Table 14).

Table 14: Percentage of stunted and severely stunted children and distribution of LAZ scores

Village	Description	At recruitment (6 - 14 months old) (n = 166)	6 months after recruitment (12 - 20 months old) (n = 148)	12 months after recruitment (18 - 26 months old) (n = 146)
Nyabula	Stunted growth (%)*	55.0	71.0	77.0
	Severely stunted (%)	30.7	33.9	26.8
	LAZ scores, mean (95% CI)	-2.4 (-2.7 to -2.1)	-2.7 (-3.1 to -2.4)	-2.7 (-3.0 to -2.4)
Kigwa	Stunted growth (%)*	21.3	57	46.0
	Severely stunted (%)	2.1	8.1	5.4
	LAZ scores, mean (95% CI)	-1.2 (-1.5 to -0.9)	-1.9 (-2.2 to -1.5)	-1.7 (-2.1 to -1.4)
Kikelelwa	Stunted growth (%)*	51.0	38	42.0
	Severely stunted (%)	12.3	7.3	18.9
	LAZ scores, mean (95% CI)	-1.8 (-2.1 to -1.5)	-1.8 (-2.1 to -1.5)	-1.9 (-2.3 to -1.6)
All	Stunted growth (%)*	44.0	55.0	56.0
	Severely stunted (%)	16.3	17.6	18.5
	LAZ scores, mean (95% CI)	-1.9 (-2.1 to -1.7)	-2.2 (-2.4 to -2.0)	-2.2 (-2.4 to -2.0)

* Severely stunted children are included

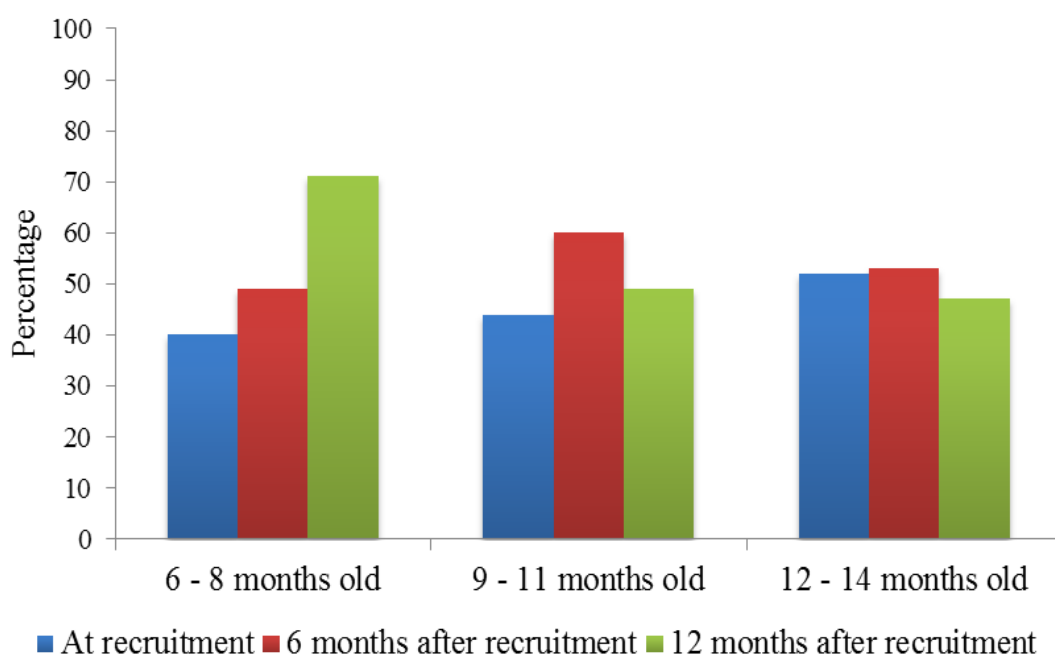


Figure 18: Percentage of child stunting by category of age at recruitment

4.13.2 Prevalence of underweight

Prevalence of underweight was 8% at recruitment and 14% both at 6 months and at 12 months after recruitment (Table 15). The overall proportion of severely underweight children ranged from 2.4% to 3.4% throughout the study. Just like stunting, there was village variation in terms of prevalence of underweight. At recruitment, the prevalence was 11% both at Nyabula and Kikelelwa village and 2% at Kigwa village. At 6 months after recruitment, the prevalence of underweight was higher at Nyabula village (21%) than Kigwa (11%) and Kikelelwa (9%). At 12 months after recruitment, the prevalence was higher at Nyabula village (30%) than 6% at Kikelelwa village and 3% at Kigwa village ($p < 0.001$) (Table 15).

At 6 months after recruitment, mean WAZ scores were significantly lower in children at Nyabula than Kikelelwa village ($p < 0.05$). These levels were also lower at Nyabula than the other villages at 12 months after recruitment ($p < 0.01$). The mean WAZ scores were significantly higher at recruitment than at 6 ($p < 0.01$) and at 12 months thereafter ($p < 0.001$).

Table 15: Percent of underweight and severely underweight children and distribution of WAZ scores

Village	Description	At recruitment (6 - 14 months old) (n = 166)	6 months after recruitment (12 - 20 months old) (n = 148)	12 months after recruitment (18 - 26 months old) (n = 146)
Nyabula	Underweight (%)*	11.0	21.0	30.0
	Severely underweight (%)	4.8	7.1	5.4
	WAZ scores, mean (95% CI)	-0.6 (-0.9 to -0.3)	1.2 (-1.5 to -0.9)	-1.3 (-1.6 to -1.0)
Kigwa	Underweight (%)*	2.0	11.0	3.0
	Severely underweight (%)	0.0	0.0	0.0
	WAZ scores, mean (95% CI)	-0.4 (-0.7 to -0.1)	-0.8 (-1.1 to -0.5)	-0.7 (-0.9 to -0.4)
Kikelelwa	Underweight (%)*	11.0	9.0	6.0
	Severely underweight (%)	1.8	1.8	1.9
	WAZ scores, mean (95% CI)	-0.4 (-0.7 to - 0.04)	-0.6 (-1.9 to -0.3)	-0.8 (-1.0 to -0.5)
All	Underweight (%)*	8.0	14.0	14.0
	Severely underweight (%)	2.4	3.4	2.7
	WAZ scores, mean (95% CI)	-0.5 (-0.7 to - 0.3)	-0.9 (-1.1 to -0.7)	-1.0 (-1.1 to -0.8)

* Severely underweight children are included

4.13.3 Prevalence of wasting, overweight as well as both stunting and underweight

Wasting was the least prevalent type of malnutrition, with up to 4% across the villages and surveys. At recruitment, the proportion of overweight children was 11%, but after 12 months, it declined to 2.7%. The wasting and overweight data are presented in Table 16. However, due to comparatively low prevalence, wasting and overweight variables were not further analysed. Proportion of children who were both stunted and underweight was 8% at recruitment, 12% at 6 months and 13.0% at 12 months after recruitment (Table 16).

Table 16: Percent of overweight, wasted, severely wasted and distribution of WLZ scores

Village	Description	At recruitment (6 - 14 months old) (n = 166)	6 months after recruitment (12 - 20 months old) (n = 148)	12 months after recruitment (18 - 26 months old) (n = 146)
Nyabula	Overweight (%)	14.5	1.8	1.8
	Wasted (%)*	2.0	2.0	2.0
	Severely wasted (%)	0.0	0	1.8
	WLZ scores, mean (95% CI)	0.9 (0.7 - 1.2)	0.2 (-0.05 - 0.5)	0.1 (-0.2 - 0.3)
Kigwa	Overweight (%)	2.1	0.0	2.7
	Wasted (%)*	4.0	3.0	0.0
	Severely wasted (%)	2.1	0.0	0.0
	WLZ scores, mean (95% CI)	0.3 (-0.04 - 0.6)	0.1 (-0.2 - 0.5)	0.3 (-0.002 - 0.6)
Kikelelwa	Overweight (%)	14.0	5.4	3.8
	Wasted (%)*	0.0	2.0	0.0
	Severely wasted (%)	0.0	0.0	0.0
	WLZ scores, mean (95% CI)	0.9 (0.6 - 1.2)	0.3 (-0.01 - 0.5)	0.3 (0.1 - 0.5)
All villages	Overweight (%)	10.8	2.7	2.7
	Wasted (%)*	2.0	2.0	1.0
	Severely wasted (%)	0.6	0.0	0.7
	WLZ scores, mean (95% CI)	0.8 (0.6 - 0.9)	0.2 (0.05 - 0.4)	0.2 (0.06 - 0.3)

* Severely wasted children are included

4.13.4 Length and weight gain by children during the 12 months of the study

The mean length gain over the 12 months of the study was 11.5 cm (range 5.4 cm – 16.7 cm). Children at Nyabula village demonstrated less mean length gain than those at Kikelelwa ($p = 0.036$). During the same period, the mean weight gain was 2.0 kg (range 0.1 - 4.1 kg), with children at Nyabula village demonstrating lower mean weight gain than children at Kikelelwa and Kigwa villages ($p < 0.001$) (Table 17).

Table 17: Length and weight gain of children over 12 months by village

Parameter	Nyabula	Kikelelwa	Kigwa	All villages	<i>p</i> -value
Length (cm)					
Mean	11.1*	12.1**	11.2	11.5	0.036
(95% CI)	(10.5 - 11.7)	(11.5- 12.6)	(10.6 - 11.8)	(11.1- 11.8)	
Range	5.4 - 16.5	5.6 - 16.7	7.1 - 14.4	5.4 - 16.7	
Weight (kg)					
Mean	1.7*	2.1**	2.3**	2.0	<0.001
(95% CI)	(1.5 - 1.9)	(1.9 - 2.2)	(2.1 - 2.5)	(1.9 - 2.1)	
Range	0.1 - 4.1	0.6 - 3.4	1.0 - 3.7	0.1 - 4.1	

*, ** Significant difference between the compared villages;

4.13.5 Tracking of childhood stunting and underweight

Tracking is defined as the maintenance of relative position in rank of behaviour over time (Twisk *et al.*, 1997) (for example infants who have been classified as stunted are likely to remain stunted into childhood or adulthood). The strength of tracking child nutritional status was determined by weighted Kappa values (k), which is interpreted as follows; $k \leq 0.20$ poor tracking; $k = 0.21 - 0.40$ fair tracking; $k = 0.41 - 0.60$ moderate tracking; $k = 0.61 - 0.80$ good tracking and $k = 0.81 - 1.0$ very good tracking (Altman, 1990). Table 18 displays percentage of children who remained in the same nutritional status or shifted from one state of nutritional status to another from age of 6 - 14 months old (at recruitment) to 18 - 26 months old (at 12 months after recruitment).

Almost 81% of the children who were stunted (both stunted and severely stunted combined) at age of 6 - 14 months, remained in the same status after one year. About 37% of children who had normal LAZ at 6 - 14 months old were stunted after one year. In the same age groups and duration, only 19% of children whose nutritional status were classified as stunted had changed to normal nutritional status. None of the severely stunted children changed to normal nutritional status and 63% of children who were classified as having normal nutritional status for LAZ scores remained within the same category.

Overall, stunting exhibited moderate strength of tracking in one year ($k = 0.43$). Similarly, tracking of stunting was moderate for children at Nyabula ($k = 0.49$) and at Kikelelwa ($k = 0.439$) but poor for children at Kigwa ($k = 0.20$). There was a significant positive correlation between baseline LAZ scores and follow up LAZ scores at 6 months after recruitment ($r = 0.86$; $p < 0.001$) and at 12 months after recruitment 12 ($r = 0.82$; $p < 0.001$).

It was noted that 62% of children who were classified as underweight and 90% of children who had normal WAZ at age of 6 - 14 months old, remained under the same nutritional status after 12 months. After one year, about 10% of children who had normal weight for age shifted to underweight and 39% of the children who were underweight shifted to normal weight. In overall, tracking of underweight status was moderate ($k = 0.41$). By village, the degree of this tracking was also moderate ($k = 0.432$) for children at Nyabula but fair ($k = 0.399$) for Kikelelwa and poor for Kigwa village ($k = -0.03$). Baseline WAZ scores correlated significantly with follow-up WAZ scores at 6 months after recruitment ($r = 0.87$; $p < 0.001$) and WAZ scores at 12 months after recruitment ($r = 0.81$; $p < 0.001$).

Table 18: Tracking of stunting and underweight status in children from age of 6 - 14 months to 18 - 26 months

Tracking nutritional status*	Nyabula village (%)	Kikelelwa village (%)	Kigwa village (%)	All (%)
Length for age status				
Stunting to stunting	96	64	71	81
Severe stunting to severe stunting	61	80	0	63
Moderate stunting to severe stunting	14	30	17	23
Severe stunting to moderate stunting	39	20	100	38
Normal to stunting	50	21	40	37
Normal to severe stunting	8	0	3	4
Stunting to normal	3	36	29	19
Severe stunting to normal	0	0	0	0
Normal to normal	50	79	60	63
<i>k</i>	0.49	0.43	0.20	0.43
<i>p</i> - value	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> = 0.06	<i>p</i> < 0.001
Weight for age status				
Underweight to underweight	100	33	0	62
Normal WAZ to underweight	22	2	3	10
Underweight to normal WAZ	0	67	100	39
Normal WAZ to normal WAZ	78	98	97	90
<i>k</i>	0.43	0.39	-0.03	0.41
<i>p</i> -value	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> = 0.567	<i>p</i> < 0.001

*normal (LAZ or WAZ \geq -2); stunting (LAZ < -2); moderate stunting (LAZ -3 to -2), severe stunting (LAZ < -3); underweight (WAZ < -2).

4.14 Association Between Mycotoxins Exposure and Child Growth

4.14.1 Association between fumonisin exposure and growth

The adjusted multiple regression analysis demonstrated that fumonisin exposure biomarker concentrations consistently showed significant negative association with LAZ scores throughout the study (Table 19). Levels of UFB₁ at recruitment were negatively associated

with LAZ scores at 6 months ($\beta = 0.19$; $p = 0.016$) and at 12 months after recruitment ($\beta = -0.20$; $p = 0.014$). The mean UFB₁ concentration at recruitment and sixth month after recruitment were negatively associated with LAZ score at 6 months ($\beta = -0.23$; $p = 0.022$) and at 12 months ($\beta = -0.30$; $p = 0.007$) after recruitment. The mean UFB₁ concentration for all three surveys was negatively associated with the LAZ scores at 12 months after recruitment ($\beta = -0.39$; $p < 0.001$). In addition, the mean UFB₁ concentration for each child from all survey periods was inversely associated with body length gain over the study period ($\beta = -0.52$; $p = 0.004$). Children were further categorised according to quartile groups of UFB₁ concentrations generated from mean exposure concentrations from the three surveys. The mean body length gain after recruitment to 12 months was 1.8 cm lower in children with mean UFB₁ concentrations in the highest quartile (above 935 pg/ml) versus the lowest quartile (below 224 pg/ml) ($p < 0.01$) (Fig. 19).

Table 19: Multiple regression analyses between fumonisin exposure biomarker levels and growth

Fumonisin biomarker	exposure	Outcome	Regression coefficient	Standard error	R²	p-Value
Exposure levels at recruitment		LAZ at 6 months after recruitment	-0.19	0.07	0.26	0.016
		LAZ at 12 months after recruitment	-0.20	0.40	0.24	0.014
Mean exposure levels at recruitment and at 6 months after recruitment		LAZ at 6 months after recruitment	-0.23	0.10	0.25	0.022
		LAZ at 12 months after recruitment	-0.30	0.10	0.23	0.007
Mean exposure levels from all three survey periods		LAZ at 12 months after recruitment	-0.39	0.10	0.27	0.000
		Length gained over 12 months	-0.52	0.18	0.24	0.004

Note: All models adjusted for village, breastfed status, maternal education, socio-economic status, protein and energy intakes. Additionally, length gain model was adjusted for sex, baseline age and baseline length

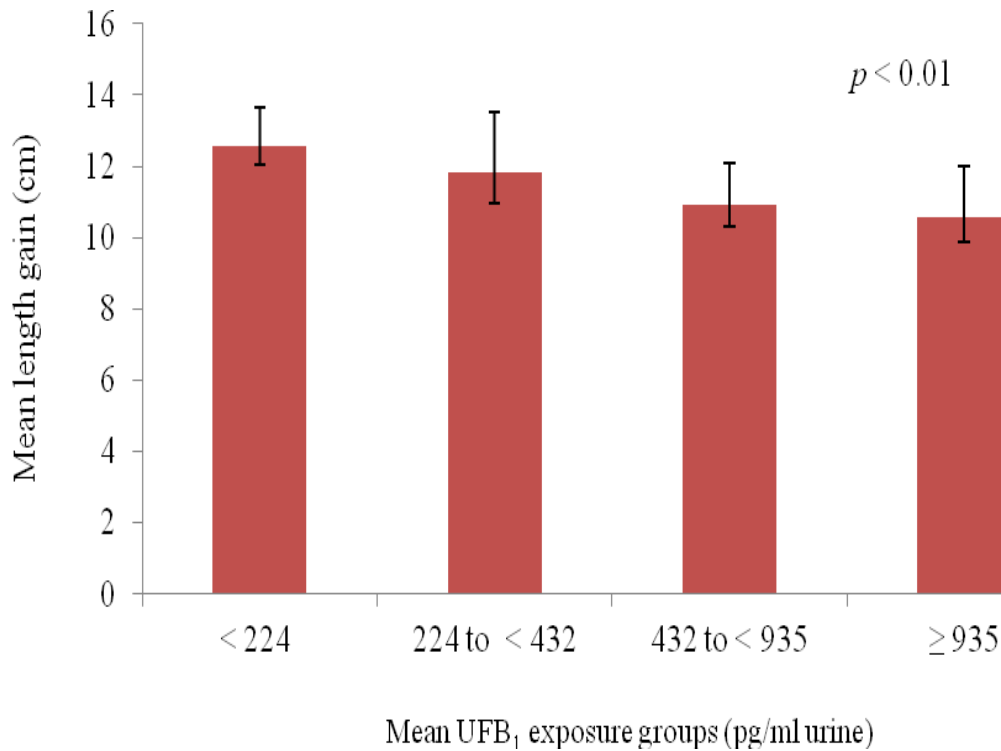


Figure 19: Mean length gain over 12 months according to UFB₁ levels

4.14.2 Association between aflatoxin exposure and child growth

In overall, there was non-significant negative association between AF-alb concentration and growth. The mean AF-alb concentration at recruitment and sixth month after recruitment were negatively associated with LAZ score at 12 months after recruitment ($\beta = -0.002$; $p = 0.998$) (Table 20). The mean AF-alb concentration for all three surveys was negatively associated with the LAZ scores at 12 months after recruitment ($\beta = -0.071$; $p < 0.493$). In addition, the mean AF-alb concentrations from all surveys was inversely associated with body length gain over the 12 months of study ($\beta = -0.326$; $p = 0.084$). Children were further categorised into quartile groups of AF-alb concentrations generated from mean exposure concentrations from all surveys' samples. Children with mean AF-alb concentrations in the lowest quartile (below 8.1 pg/mg) were found to gain more length (mean 11.8 cm) compared to the children in the highest exposure quartile (above 41.13

pg/mg), who had mean length gain of 10.5 cm ($p < 0.05$) (Fig. 20). The overall mean length gain during the 12 months of study was 11.5 cm.

Table 20: Multiple regression analyses between aflatoxin exposure biomarker levels and growth

Aflatoxin exposure biomarker	Outcome	Regression coefficient	Standard error	R ²	p-Value
Exposure levels at recruitment	LAZ at 6 months after recruitment	0.069	0.092	0.19	0.456
	LAZ at 12 months after Recruitment	0.078	0.089	0.19	0.385
Mean exposure levels at recruitment and at 6 months after recruitment	LAZ at 6 months after recruitment	0.027	0.087	0.19	0.756
	LAZ at 12 months after recruitment	-0.002	0.085	0.18	0.998
Mean exposure levels from all three survey periods	LAZ at 12 months after recruitment	-0.071	0.102	0.18	0.493
	Length gain over 12 months	-0.326	0.187	0.16	0.084

Note: All models adjusted for village, breastfed status, maternal education, socio-economic status, protein and energy intakes. Additionally, length gain model was adjusted for sex, baseline age and baseline length.

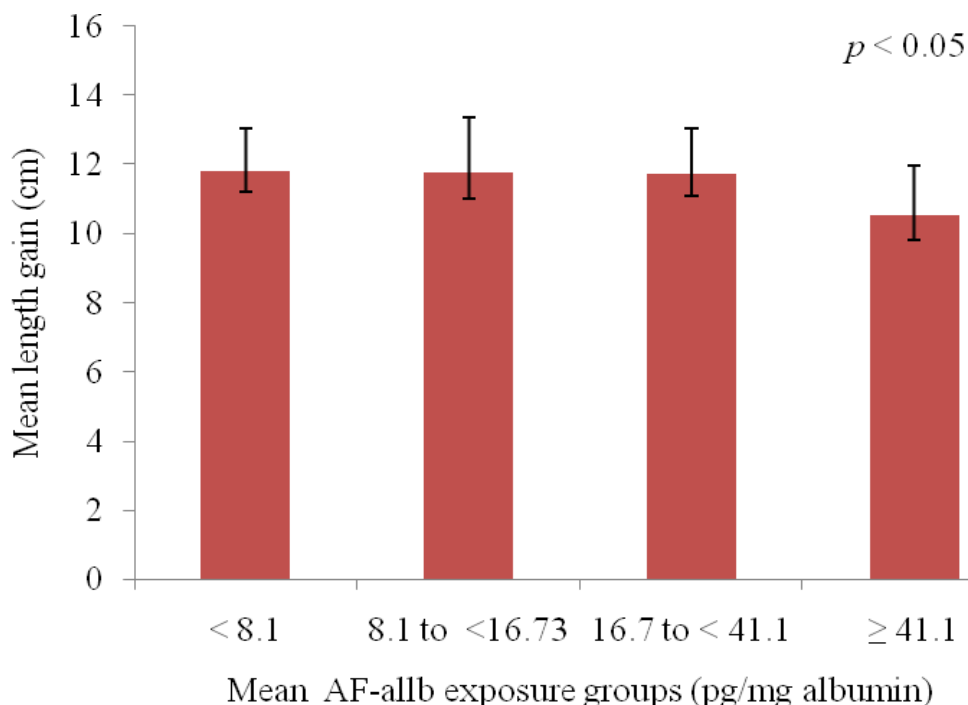


Figure 20: Mean length gain over 12 months according to AF-alb levels

4.15 Determinants of Stunting

Results of binary logistic regression between stunting and associated factors are shown in Table 21 with the odds ratio and 95% CI. Unadjusted variables that were significantly associated with child stunting included low birth weight (< 2500 g) [OR: 5.4 (1.90 - 15.63); $p = 0.002$], early age at start of complementary feeding [OR: 2.6 (1.26 - 5.29); $p = 0.009$], morbidity during the preceding three months before a survey [OR: 2.6 (1.30 - 5.04); $p = 0.006$] and low feeding frequency (less than 3 meals per day) [OR: 2.8 (1.40 - 5.65); $p = 0.004$]. The odds of stunting were higher when children were at 18 - 26 months old [OR: 1.63 (1.04 - 2.55), $p < 0.05$] compared to when they were at age of 6 - 14 months old. Higher odds of stunting were observed in children from low SES households than the medium [OR: 2.5 (1.23 - 5.30); $p = 0.012$] or high SES households [OR 3.2 (1.10 - 9.31); $p = 0.032$]. Child residence was found to be associated with stunting. Children from Nyabula village (Iringa region) had increased odds of being stunted compared to the children at Kikelelwa village (Kilimanjaro region) [OR: 4.7 (2.04 - 10.65); $p < 0.001$] and Kigwa village (Tabora region) [OR: 3.9 (1.59 - 9.53); $p = 0.003$]. Being stunted at recruitment (age 6 to 14 months) significantly increased the likelihood of a child being stunted after one year OR: [7.5 (3.47 - 16.25); $p < 0.001$]. High concentrations of UFB₁ were associated with increased likelihood of stunting OR: [2.5 (1.26 - 4.8); $p < 0.01$].

A variable which remained significant in multivariate logistic analysis was; child age; with children at 18 - 26 months old having increased risk of stunting compared to when they were at 6 - 14 months [OR: 1.7 (1.03 - 2.68), $p = 0.039$]. Other significant variables were village; with children at Nyabula having increased odds of stunted compared to Kikelelwa [OR: 6.3 (1.3 - 30.2); $p = 0.022$], past record of stunting [OR: 14.03 (4.88 - 40.34); $p < 0.001$] and high exposure to fumonisin (i.e UFB₁ concentration above median) [OR: 2.1 (1.0 - 4.4)].

Table 21: Odds ratio of predictor variables for stunting (LAZ < -2) from binary logistic regression

Variable	Stunting			Stunting		
	Crude OR	(95% CI)	<i>p</i> -value	Adjusted OR	(95% CI)	<i>p</i> -value
Sex	1.35	0.69 - 2.60	0.371			
Low birth weight	5.4	1.90 - 15.63	0.002			
Age: 6 - 14 versus 18 - 26 months	1.63	1.04 - 2.55	0.032	1.66	1.03 - 2.68	0.039
Not breast feeding	1.9	0.47 - 7.65	0.368			
Age of complementary feeding	2.6	1.26 - 5.29	0.009			
Morbidity	2.6	1.30 - 5.04	0.006			
Having less than three meals of complementary foods per day	2.8	1.40 - 5.65	0.004			
Socio-economic status:-						
Low versus medium	2.5	1.23 - 5.30	0.012			
Low versus high	3.2	1.10 - 9.31	0.032			
Village: Nyabula versus Kikelelwa	4.7	2.04 - 10.65	0.000	6.26	1.30 - 30.18	0.022
Nyabula versus Kigwa	3.9	1.59 - 9.53	0.003			
Having less than six food varieties per week	2.32	1.18 - 4.56	0.014			
Previous stunting	7.5	3.47 - 6.25	0.000	14.03	4.88 - 40.34	0.000
Household size	0.9	0.82 - 1.01	0.090			
Maternal with no formal education	0.9	0.29 - 2.83	0.860			
Maternal age	0.9	0.93 - 1.03	0.386			
Mother living single	1.8	0.79 - 4.05	0.158			
AF-albumin concentration above median	1.1	0.50 - 2.36	0.827			
Urinary FB ₁ concentration above median	2.5	0.26 - 4.80	0.008	2.1	1.0 - 4.4	0.049

4.16 Determinants of Underweight

Unadjusted factors that were significantly associated with child underweight included low birth weight [OR: 8.8 (3.57 - 21.68); $p < 0.001$], not breast feeding [OR: 1.86 (1.05 - 3.29); $p = 0.033$] and low feeding frequency [OR: 2.8 (1.55 - 5.10), $p = 0.001$] (Table 22). Village of residence was also another factor, with children at Nyabula having higher odds of underweight than Kikelelwa [OR: 2.8 (1.45 - 5.44); $p = 0.002$] and Kigwa [OR: 5 (2.03 - 12.3); $p < 0.001$]. High concentrations of AF-alb were associated with increased likelihood of underweight OR: [2.9 (1.05 - 7.93); $p < 0.01$].

Variables which remained significant in multivariate logistic model were; not breast feeding [OR: 2.01 (1.10 - 3.69), $p = 0.023$], few meals (i.e <3) per day [OR: 2.24 (1.00 - 4.99), $p = 0.050$] and village of residence; with children at Nyabula demonstrating higher odds for underweight than Kigwa [OR: 4.31 (1.70 - 10.9), $p = 0.002$].

Table 22: Odds ratio of predictor variables for underweight (WAZ < -2) from binary logistic regression

Variable	Underweight			Underweight		
	Crude OR	(95% CI)	<i>p</i> -value	Adjusted OR	(95% CI)	<i>p</i> -value
Sex	1.53	0.87 - 2.69	0.138			
Low birth weight	8.80	3.57 - 21.68	0.000			
Age: 6 - 14 versus 18 - 26 months	1.82	0.89 - 3.73	0.100			
Not breast feeding	1.86	1.05 - 3.29	0.033	2.01	1.10 - 3.69	0.023
Age of complementary feeding	1.05	0.88 - 1.25	0.581			
Morbidity	0.58	0.31 - 1.05	0.070			
Having less than three meals of complementary foods per day	2.80	1.55 - 5.10	0.001	2.24	1.00 - 4.99	0.050
Socio-economic status:						
Low versus medium	1.93	1.06 - 3.51	0.031			
Low versus high	2.66	0.90 - 7.89	0.076			
Village: Nyabula versus Kikelelwa	2.80	1.45 - 5.44	0.002			
Nyabula versus Kigwa	5.00	2.03 - 12.3	0.000	4.31	1.70 - 10.9	0.002
Having less than six food varieties per week	1.60	0.90 - 2.88	0.100			
Household of size	0.87	0.78 - 0.99	0.032			
Maternal with no formal education	0.18	0.02 - 1.34	0.094			
Maternal age	0.99	0.95 - 1.04	0.870			
Mother living single	0.65	0.31 - 1.37	0.256			
AF-albumin concentration above median	2.9	1.05 - 7.93	0.040			
Urinary FB ₁ concentration above median	0.57	0.22 - 1.47	0.242			

CHAPTER FIVE

5.0 DISCUSSION

Tanzania, like other tropical countries, has hot and humid environments which are conducive for aflatoxin and fumonisin contamination of cereals and groundnuts. These mycotoxins are associated with chronic and acute toxicological manifestations in humans and the risk is particularly high in children. High mycotoxin levels have been detected in a variety of food stuffs, mainly maize and groundnuts, which are the main ingredients of children complementary food. The present study was conducted to assess the magnitude and distribution of dietary exposure of young children to aflatoxin and fumonisin by using biomarker of mycotoxin exposure, assess child growth and its association with exposure to the mycotoxins.

5.1 Status of Aflatoxin Exposure in Children

This study found high prevalence of biomarkers of aflatoxin exposure (aflatoxin albumin adducts) in the blood of children, indicating that dietary exposure to this potent toxin is prevalent from an early age as well as chronic. These observations imply that contamination of food by aflatoxin is widespread in Tanzania as earlier reported in maize (Kimanya *et al.*, 2008a; Manjula *et al.*, 2009; Mboya *et al.*, 2011; Kamala *et al.*, 2015), groundnuts (Abt Associates Inc., 2013), cassava (Manjula *et al.*, 2009), cured fish (Mugula and Lyimo, 1992) and locally processed cereal based complementary foods (Rushunju *et al.*, 2013). The findings from this study therefore suggest the rationale for implementation of measures to control aflatoxin contamination in the food chain and prevent exposure of human to the toxin in order to protect public health.

The overall geometric mean concentrations of AF-alb (at 95% CI) found in the children across the three survey periods; 4.7 (3.9 - 5.6) pg/mg albumin at recruitment, 12.9 (9.9 - 16.7) pg/mg albumin at 6 months and 23.5 (19.9 - 27.7) pg/mg albumin at 12 months after recruitment were lower than the mean concentrations reported in children from other parts of Africa. For instance, AF-alb levels of 32.8 pg/mg were reported in children aged nine months to five years old in Togo and Benin (Gong *et al.*, 2003) and 31.1 to 119.3 pg/mg in 16 - 37 month old children from three villages in Benin (Gong *et al.*, 2004). Similarly, the overall concentrations from this study (except at 12 months after recruitment) were lower than 22.3 pg/mg reported in Gambia (Turner *et al.*, 2003) and 206.5 pg/mg and 73.2 pg/mg reported in two neighbouring villages in Kenya (Gong *et al.*, 2012). This difference is partly because the other studies included children at an older age and it is evident that exposure to aflatoxin increases with age during the complementary feeding period as demonstrated by Gong *et al.*, (2003). Different geographical location, even within the same country and different season, when the study took place are key determinants of exposure as demonstrated in many studies in Africa (Gong *et al.*, 2003, 2012; Turner *et al.*, 2003, 2005, 2007). However, levels detected in children at Kigwa village alone during the second and the third surveys were higher than levels reported in the compared studies, except for the Kenya study which involved much older children (aged 6 - 17 years) (Gong *et al.*, 2012). This could suggest that in Kigwa village, there is high contamination of aflatoxin in food and high intake of contaminated food compared to the other areas. Furthermore, the mean levels of 4.7 (3.9 - 5.6) detected at recruitment in the present study when children were 6 - 14 months old were less than 9.7 (8.2 - 11.5) pg/mg albumin detected in children aged 0 - 3 years in Uganda (Asiki *et al.*, 2014) and 8.7 pg/mg reported in 16 week old infants in Gambia (Turner *et al.*, 2007). The disparities in levels of aflatoxin exposure can be

attributed to factors such as level of aflatoxin contamination in food together with amount of food that is consumed, age of subject and duration of exposure as well as individual variations in toxicokinetics of the toxin as earlier reported (Wild *et al.*, 2000ba). Such factors should be taken into consideration in planning and implementation of strategies to protect children from exposure to mycotoxin.

5.2 Status of Fumonisin Exposure in Children

In line with high level of exposure to aflatoxin, the study further revealed high prevalence and levels of exposure of children to fumonisin, demonstrating that they are at risk of dietary exposure to more than one type of mycotoxin. Acute exposure of children to multiple mycotoxins predisposes them to more health risks since there is emerging evidence suggesting that the two toxins altogether may have additive or synergistic toxicological effects in humans or animals (Capriotti *et al.*, 2012; Berthiller *et al.*, 2013; Siloto *et al.*, 2013; Abdel-Wahhab *et al.*, 2014). In this study, prevalence of fumonisin exposure was very high. The toxin was detected in urine samples from 96% to 100% of all subjects throughout the three surveys. Biomarker studies to assess fumonisin exposure in children are limited to the study conducted in Cameroon (Ediage *et al.*, 2013), whereby the mean UFB₁ concentration was 2960 (60 - 48 000) pg/ml urine. In the present study, mean concentrations of UFB₁ (at 95% CI) detected throughout the three sampling periods were 313.9 (257.4 - 382.9) pg/ml urine at recruitment, 167.3 (135.4 - 206.7) pg/ml urine at 6 months and 569.5 (464.5 - 698.2) pg/ml urine at 12 months after recruitment. These values were lower than 2960 (60 - 48 000) pg/ml urine observed in the Cameroonian children (Ediage *et al.*, 2013). In addition, the levels of UFB₁ detected at recruitment and at 12 months after recruitment from the present study were higher than 225 (114 - 350) pg/ml and 109 (85 - 138) pg/ml previously reported at baseline and after intervention respectively, in South African female adults (Van der

Westhuizen *et al.*, 2011a). However, the UFB₁ levels detected at 6 months after recruitment from the present study [167.3 (135.4 - 206.7) pg/ml] were lower than the levels of the compared study at baseline but higher than the levels of the compared study, after intervention (Van der Westhuizen *et al.*, 2011a). Levels across the three surveys were higher than levels previously reported in adults in Mexico, which ranged from 35.0 (18.8 - 65.2) pg/ml to 147.4 (87.6 - 248) pg/ml (Gong *et al.*, 2008b). Differences in UFB₁ concentrations demonstrated between the compared studies may be contributed to several factors such as differences in age, gender, level of fumonisin contamination in maize, quantities of maize consumption, methods used for food preparation and processing and duration of exposure. In addition, different methodologies may also contribute to the discrepancy between the compared studies (Solfrizzo *et al.*, 2013). Urinary excretion of FB₁ in adults reported in a previous study in South Africa was estimated to be 0.075% of the fumonisin intake (Van der Westhuizen *et al.*, 2011a). However, a recent controlled study on American adults consuming maize of known concentration of fumonisin B₁ contamination demonstrated that on average, 0.5% of the toxin intake is excreted in urine (Riley *et al.*, 2012). It is also possible that FB₁ excretion in adults may not be comparable to that of young children due to differences in physiological mechanisms that may influence excretion of FB₁ in urine. The variations in the UFB₁ levels observed across populations substantiate the need for further investigation studies to examine the physiological mechanism of FB₁ excretion in urine among different age or ethnic groups.

5.3 Village Variation in Aflatoxin Exposure

During each survey period, there was variation in prevalence and magnitude of aflatoxin exposure between the three studied villages. The levels of AF-alb were higher

in children from Kigwa village (Tabora region), followed by Nyabula village (Iringa region) and lowest at Kikelelwa village (Kilimanjaro region). For instance, during the second survey, there was a 12-fold difference in mean AF-alb levels between Children at Kigwa village and Kikelelwa village. Children from Kikelelwa village on average eat less maize on a daily basis compared to the other villages because of availability of other food options, mainly bananas and potatoes, which are non-susceptible to aflatoxin contamination. This observation is supported by findings from a study conducted in Uganda in which lower AF-alb levels were detected in people consuming banana and less maize compared to those who consumed more maize (Asiki *et al.*, 2014). This observation suggests that dietary diversification is one of the key measures for protecting exposure of humans to aflatoxins. Levels of AF-alb detected from the studied children were significantly higher among children who were reported to consume groundnuts than those who did not consume groundnuts as shown in Kigwa village where levels of AF-alb were the highest. Kigwa village is located in Tabora region, which leads in production of groundnuts in Tanzania (URT, 2005).

Additionally, it is also important to recognise that the three studied villages are from different agro-ecological zones, with varying climatic conditions, harvest practices, food preparation methods and food storage, which could lead to different levels of aflatoxin contamination and exposure between them. Geographical difference in aflatoxin exposure has also been widely reported in several other studies including Kenya (Gong *et al.*, 2012; Yard *et al.*, 2013), Togo and Benin (Gong *et al.*, 2003) and Uganda (Asiki *et al.*, 2014).

5.4 Village Variation in Fumonisin Exposure

There was huge variation observed in levels of fumonisin exposure in children between the three villages studied. The mean exposure levels in children at Kigwa village were higher than those at Kikelelwa by four-fold, at 6 months after recruitment. Similarly, at 12 months after recruitment, the levels were higher by three-fold in children at Nyabula village than Kikelelwa. This village variation in fumonisin exposure could be attributed to variations in climatic conditions between the villages, which can differently influence growth of the fungi of *Fusarium* species. Other studies have also documented geographical variation in fumonisin contamination in maize (Sun *et al.*, 2007; Kimanya *et al.*, 2008b; Wei *et al.*, 2013).

5.5 Influence of Maize Intake and Maize Processing on Mycotoxin Exposure

This study was conducted in communities where the households consume mainly the food produced from their own farms or purchased from local markets. Levels of UFB₁ in this study correlated significantly with quantities of maize intake by children, suggesting that this biomarker is a good indicator of fumonisin intake by this group of the population. Consistently higher maize intake and higher levels of UFB₁ were found in children from villages of Kigwa and Nyabula than Kikelelwa due to maize being dominant food consumed. In Kikelelwa, there was a bit of diversification on the types of foods. These observations suggest that maize in the studied areas was contaminated with fumonisins as reported in the previous studies (Kimanya *et al.*, 2007, 2008a; Kamala *et al.*, 2015). Since maize was the main ingredient in children complementary as well as the family foods, intervention targeted to manage mycotoxins contamination in this food crops and to promote dietary diversification should be considered as key measures to protect children from exposure to mycotoxins. Studies conducted on adults in Mexico (Gong *et al.*, 2008b) and China (Xu *et al.*, 2010) observed higher

mean UFB₁ levels in participants from the high maize consumption group compared to the low maize consumption group.

Maize processing mainly de-hulling (a milling technique to remove the pericarp and germ of maize) reduced the levels of exposure to fumonisin and aflatoxin in Kigwa village. Children reported to have consumed dehulled maize were detected with lower levels of the aflatoxin and fumonisin exposure biomarkers compared to those who consumed whole kernel maize. This finding suggests the potential of maize processing methods particularly de-hulling as one of possible measures for reducing exposure of children to mycotoxins. Dehulling has been reported to reduce levels of aflatoxin and fumonisin contamination in maize; for example 34% of aflatoxin and 29% of fumonisin contamination levels in raw maize have been detected in maize hulls and germ (Fandohan *et al.*, 2005b). The outer layers of maize kernels have been therefore reported to be the parts most susceptible to fungal attack and mycotoxin accumulation (Siwela *et al.*, 2005).

5.6 Impact of Season on Aflatoxin and Fumonisin Exposure

There was a difference in mycotoxin exposure levels between the three survey periods, a pattern which reflects the combination of increased consumption of contaminated food and the seasonal variation of mycotoxins contamination and the subsequent exposure. In the present study, the first survey was conducted during the maize harvest season (i.e between June and July) when people were consuming fresh maize. The second survey was at 6 months after the first survey (i.e between January and February), when people were consuming maize which has been stored for about 6 - 7 months. The third survey took place during the following year's season of maize harvest (i.e between June and July). The mean AF-alb levels increased progressively, with each survey period demonstrating higher

levels than the previous one. Aflatoxin contamination occurs at both harvest and storage stage due to the nature of growth and spreading of the fungi, which produces the toxin. The aflatoxin producing fungi occur in developing crop in the field and later proliferate in storage if conditions are favourable (Gallo *et al.*, 2016). For instance, infestation of stored maize by insects leads into production of moisture and temperature, which in turn provide favourable condition for growth of aflatoxins producing fungi (Chulze, 2010). The observed increase in aflatoxin exposure would be expected at the second survey (i.e at 6 months after recruitment) when a high proportion of stored maize was being consumed. This observation is supported by findings from other studies which have shown that concentration of aflatoxins in food tends to increase with time during storage (Tédihou *et al.*, 2012; Hell *et al.*, 2003) and therefore increase post-harvest toxin contamination. The observation from the present study therefore reinforces the importance of improved food storage for reducing post harvest contamination.

Fumonisin exposure levels were significantly higher at recruitment (during maize harvest seasons), than at the second survey (6 months post-harvest) when stored maize was consumed. Fumonisin contamination has been recognised as predominantly a pre-harvest (field stage) problem (Turner, 2014) and contamination levels in food are not likely to increase during storage in the same way that aflatoxin levels do. Reduced fumonisin exposure observed during the second survey could be explained from the fact that maize stocks are usually low in the subsistence farming families at 6 months after harvest, which is associated with reduced maize intake and increased reliance on other types of seasonally available local foods. Again, the fumonisin exposure became much higher at the last survey (i.e at 12 months after recruitment which was also another harvest season), than the previous year's harvest season (i.e at recruitment). The possible explanation for the increased concentration of the fumonisin exposure biomarker during the last survey is

increased maize intake associated with increased age of the children hence more reliance on complementary foods than breast feeding together with availability of adequate maize supply during the harvest season as well as seasonal variation.

5.7 Impact of Age on Aflatoxin and Fumonisin Exposure

The present study observed a positive correlation between the levels of mycotoxin exposure biomarkers with age of children. This observation demonstrates that as children grew-up, they were exposed to higher levels of mycotoxin due to increased amounts of complementary food consumed. The strength of association between levels of aflatoxin exposure biomarker and age was different at each survey period; at recruitment (age 6 - 14 months), the association was positive but did not reach statistical significance and this could be attributed to intake of small amount of complementary foods associated with breast feeding as high proportion (93%) of children were still breastfed during the survey period. At 6 and at 12 months after recruitment, the positive correlation between aflatoxin exposure biomarkers and age became significant due to increased amount of food intake as the children were growing up and depended mainly on complementary food and not breast milk for their nutritional intake. Additionally, the strong positive correlation between AF-alb levels and age observed at 6 months after recruitment ($r = 0.326$; $p < 0.001$) could be attributed to increased concentration of aflatoxin contamination in the stored food due to impact of storage condition. Positive correlation between levels of mycotoxin exposure and age observed in the present study align with a previous study in Benin, which showed that age of a child contributes significantly to the levels of AF-alb, largely due to increased quantities of complementary food intake as the child grows older (Gong *et al.*, 2004). However, this age-dependent effect was not seen in older children at age of 6 - 17 years in Kenya (Gong *et al.*, 2012). The results further indicate that age is a surrogate indicator of increased food intake and reduced breast feeding of a child (Gong *et al.*, 2003).

5.8 Breastfed Status and Extent of Exposure to Mycotoxins

Continued breast feeding during complementary feeding period significantly contributed to decreased exposure of children to mycotoxins. Since both aflatoxin and fumonisin exposure shared the same trend i.e. both were higher in non-breastfed than in breastfed children, this observation suggests that consumption of contaminated complementary food is the driving force behind the increased exposure. In this study, amount of maize (i.e mycotoxins susceptible food) intake was higher among the non-breastfed children than those who were breastfed, suggesting that promoting exclusive breast feeding during the first 6 months may protect children from early exposure to mycotoxins from complementary foods. In addition, continued breast feeding during complementary feeding as recommended by WHO (WHO, 2003) may limit the extent of exposure because it tends to reduce the amount of complementary food intake and therefore total exposure. Thus, breast feeding not only has nutritional advantage, but also serves to reduce and delay high exposure to mycotoxins at an early age. However, while promoting breast feeding, it is also important to protect maternal exposure to mycotoxins so as to avoid contamination of breast milk due to possible carry-over of the toxins from consumption of contaminated food by a mother into the breast milk (Khlangwiset *et al.*, 2011; Magoha *et al.*, 2014a, b). Lower levels of mycotoxin exposure in breastfed children than in non-breastfed children have also been reported in other places such as Togo, Benin (Gong *et al.*, 2003) and Egypt (Shouman *et al.*, 2012).

5.9 Children's Nutritional Status and the Determinant Factors

It is evident from the current study that in line with high prevalence of aflatoxin and fumonisin exposures, stunting and underweight were also prevalent among the studied children. Stunting, which is an indicator of prolonged nutritional deprivation and

cumulative growth deficit was at the highest prevalence demonstrating to be a significant public health burden. High rate of stunting observed throughout this study demonstrates high vulnerability to malnutrition in the young children. Of another fundamental concern is severe stunting and occurrence of both stunting and underweight in some of the children, since this condition is detrimental to child health.

5.9.1 Association between exposure to fumonisin and child growth

Fumonisin exposure appeared to be a possible factor in slowed child growth as levels of UFB₁ concentration were negatively associated with growth. The negative association of fumonisin exposure with child growth was consistent when the exposure was measured either at recruitment or as the mean of two or three survey periods. Observations from the present study align with evidence regarding potential mechanisms of effect for fumonisin based on experimental studies, which suggest that this toxin could contribute to growth impairment. Fumonisin disrupts sphingolipid metabolism in the gastro-intestinal tract of mice (Enongene *et al.*, 2000), damages intestine permeability in experimental studies (Lallès *et al.*, 2009), decreases food consumption and body weight in piglets (Dilkin *et al.*, 2003) and causes diarrhoea and abdominal pain in humans (Bhat *et al.*, 1997). These findings on the effects of fumonisin have raised concern that the toxin may induce intestinal enteropathy, a subclinical condition of the small intestine, characterised by reduced absorptive capacity and increased intestinal permeability, therefore mediating stunting (Smith *et al.*, 2012). While fumonisin exposure was negatively associated with growth in this study, it is also important to recognise that there are other factors which affect growth and could confound the association between UFB₁ levels and growth impairment. In view of this, further research is needed to establish the possible causal mechanisms that could link growth impairment with fumonisin exposure. Since the present study is the first one to report a negative association between fumonisin exposure from

biomarker assessment and child growth, its findings could not be compared with similar studies. However a previous study (non biomarker) in Tanzania reported that infants exposed to fumonisins at levels above the PMTDI of 2 µg/kg bw/day were significantly shorter and lighter than those who were exposed to lower levels (Kimanya *et al.*, 2010).

5.9.2 Association between exposure to aflatoxin and child growth

In this study, mean aflatoxin concentration from the three survey periods showed a negative association with child growth, consistent with findings reported in earlier studies in Togo and Benin (Gong *et al.*, 2002, 2003) and Egypt (Shouman *et al.*, 2012). The association however was not statistically significant in this study. The overall mean AF-alb across the three sampling times in this study was less than those reported in children of similar age in the studies cited above, which may explain the non-significant nature of the observed association. The possible mechanism for association of aflatoxin exposure and growth impairment is inhibition of protein synthesis due to binding of AFB₁ with DNA, RNA and proteins and therefore cause interference with enzymes and substrates that are required for processes involved in protein synthesis (Bbosa *et al.*, 2013a). This mechanism could explain the increased prevalence of child stunting shown in this study. The finding from the present study therefore does not negate the previously established significant association of aflatoxin exposure with poor child growth in other studies. Indeed, these findings further demonstrate the rationale for studies to investigate and provide information on the possible circumstances under which exposure may impair child growth. It is likely that effects of mycotoxin exposure on growth could also be determined by a number of factors (individually or combined), such as age or specific critical period at exposure (prenatal and/or post natal), exposure dose, exposure duration, genetics, health or nutritional status. The findings on association of mycotoxin exposure and growth impairment seen in this study suggest that intervention measures to prevent exposure of

children to mycotoxins should also be considered as one of the key initiatives for improving child health and nutrition.

5.9.3 Tracking of nutritional status over 12 months

The present study has shown impaired child growth tracked over time. For instance, the majority (81%) of children who were found to be stunted at age between 6 to 12 months were still stunted after one year. Stunting being a result of chronic nutrition deficit, which progresses slowly before it manifests and peaks, the trend found in this study exhibits that most of the children who were stunted at early age were most likely to be continuously stunted one or more years later. This demonstrates that it can be a challenge for malnourished children to recover and improve their nutritional status over time if the same practices continue. The correlations of baseline LAZ to the follow-up LAZ and of baseline WAZ to the follow-up WAZ were significantly high ($p < 0.001$), hence further suggesting that children who were stunted or underweight remained under the same status after one year. These findings further suggest the importance of follow up on child growth performance, starting from prenatal to early infancy in order to identify the critical period at which child growth starts to falter and the possible associated predictors. Based on the status of nutrition at an early age, children who might be stunted or underweight later in life can be identified and intervened.

The study further observed some children who were not stunted at recruitment but when assessed after one year, they were found to be stunted or severely stunted. This suggests that there was failure to maintain adequate nutritional requirements as the children were growing. A similar trend was also seen for the index of underweight. Likewise, there was a dramatic decrease in prevalence of overweight from about 11% for infants and children at 6 - 14 months old to around 3% at one year later, therefore demonstrating that there was

failure to maintain adequate growth for some of the children. Early onset of poor nutritional status is observed as important determinant of malnutrition throughout childhood. Among children who were severely stunted at recruitment, 37.5% of them shifted to moderate stunting while none of them changed to the normal state (non-stunting), suggesting that catch up growth can be more difficult for children with severe type of malnutrition. This further justifies the need to monitor child growth more frequently, identify factors responsible for continuous growth faltering and most important to implement timely actions to treat and prevent under-nutrition. These observations are similar to those made by other researchers in a study done in rural areas of South Africa (Mamabolo *et al.*, 2005) and Democratic Republic of Congo (Kismul *et al.*, 2014), which reported risk of early age under-nutrition on stunting or underweight at later childhood.

5.9.4 Effect of child age on stunted growth and underweight

The age of a child was another important determinant factor for stunting observed in this study. At age of 6 to 14 months when children were recruited for the study, prevalence of stunting was lower than the status one year later, when they were at age of 18 to 26 months. Similarly, levels of exposure to aflatoxin and fumonisin increased as child age increased and have demonstrated negative association with growth. This relationship can be explained on the basis of increased child age as an indicator for feeding transition, which involves introduction of complementary foods and less dependence on breast feeding. Introduction to complementary food may expose the children to various food contaminants such as microbial and chemical contaminants such as mycotoxins, which are likely to affect immunity and integrity of the intestinal tract since the toxins may impair the intestinal barrier or absorption capacity (Smith *et al.*, 2012). In addition, the complementary food being introduced may be inadequate in terms of quantity and quality to cope with increased child's demand for energy and nutrients requirements for optimal growth. Previous studies

have demonstrated complementary feeding period to be a stage whereby children are highly susceptible to environmental enteropathy, a common health problem in the sub-Saharan region due to chronic inflammatory pathology in the gut (Campbell *et al.*, 2004). It features damaged intestinal permeability and reduced nutrients absorption, hence increase susceptibility to health effects such as impairment of immune function and increased child malnutrition, specifically stunting (Smith *et al.*, 2012). Similarly, epidemiological studies have shown that aflatoxin exposure increases with age (Gong *et al.*, 2003) and contributes to child stunting (Gong *et al.*, 2008a). The association of increased age with increased stunting found in the present study has also been reported in other studies (Victora *et al.*, 2010; Abubakar *et al.*, 2012; Fotso *et al.*, 2012).

5.9.5 Impact of village of residence on child nutritional status

There was considerable variation in prevalence of malnutrition among children across the three villages studied. Although under-nutrition was demonstrated in all villages, the risk of stunting and underweight as well as the tracking of the same nutritional status were higher among children at Nyabula village in Iringa region relative to those at Kikelelwa village in Kilimanjaro region and Kigwa village in Tabora region. Although not adequately investigated in this study, factors such as those related to local food habits, child feeding practices, food security and feeding knowledge may be attributed to the observed differences. For instance, in this study child feeding frequency per day and varieties of complementary foods were generally low in Nyabula village compared to Kikelelwa or Kigwa villages. Likewise, children from this village had lower mean birth weight than children in the other two villages, a factor which is detrimental to child growth (Arifeen *et al.*, 2001). This finding suggests that Nyabula village is at greater risk of childhood under-nutrition. The further suggests that efforts to address malnutrition should be focused and prioritised to communities which are at higher risk of childhood undernutrition. Iringa

region (where Nyabula village is situated) has been reported as one of the four regions in Tanzania with the highest levels of stunting among children of less than five years old, at 52% (NBS and ICF Macro, 2011). Overall, findings on high prevalence of growth impairment observed in this study suggest the need for local based strategies for improving children's nutritional status during the complementary feeding period. Other studies have also documented place of residence as important determinant of child malnutrition (Fotso, 2006; Pathak and Singh, 2011; Debnath and Bhattacharjee, 2014). Although poor household socio-economic status is widely known to be associated with poor nutrition, multivariate regression results did not confirm this association. However, it is not surprising since most of the rural households rely heavily on their own food production compared to urban households where food constitutes large percentage of total household expenditures (Fotso, 2006).

5.9.6 Influence of feeding frequency on nutritional status

Multivariate analysis showed that low feeding frequency was another important risk factor for underweight but not for stunting. Impact of low feeding frequency can be stronger in underweight, which is acute in nature compared to stunting, which progresses slowly before it is detected. In this study, children who had less than three meals per day were more likely to be underweight than those who had three or more meals. Feeding frequency is a proxy for adequacy of complementary feeding. The recommended number of feeds of complementary foods for children from age of 9 months is 3 - 4 times per day plus nutritious snacks offered 1 - 2 times per day. However, higher frequency may be required depending on energy density, amount of meal and breast feeding status (Dewey, 2002). About half of the children (43% - 49%) had less than the recommended minimum number of meals per day during the survey period. Inadequate dietary intake is one of the immediate causes of malnutrition (UNICEF, 2009) and this is also common in rural

Tanzania population where dietary intake lacks nutritional diversity due to high dependency on locally home-produced foods (URT, 2010). Inadequate food availability and low level of education among the mothers could also be responsible for inadequate feeding, which would be worth to examine in other studies. Of the other factors examined, early termination of breast feeding was associated with underweight when the children were at age of 6 to 14 months but not afterwards. Non breast feeding affects child nutritional status particularly around the age at which complementary feeding is introduced. These results reinforce the reality that child under-nutrition is a problem of inappropriate and/or insufficient feeding practices. This observation further suggests that education on appropriate child feeding practices and dietary interventions are crucial for mitigating child malnutrition especially in rural communities. Association between childhood undernutrition and inadequate feeding practices has also been documented in other studies (Arifeen *et al.*, 2001; Meshram *et al.*, 2012).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The main objective of the present study was to determine the magnitude of dietary exposure to aflatoxin and fumonisin and its association with growth in young children. This study has provided for the first time, the detailed quantitative exposure information on aflatoxin and fumonisin in young children in Tanzania through biomarker measurements. Detection of aflatoxin albumin adducts in plasma and fumonisin in urine samples from young children shows that ingredients of complementary foods and family foods consumed by the studied children are contaminated with the toxins that are associated with health risks. These findings, further show that children are exposed to aflatoxins and fumonisins and are at risk of suffering from health effects such as cancers, immune suppression, growth impairment and neural tube defects, which are known to be associated with these mycotoxins. Exposure of children to mycotoxins is of particular concern since they are highly vulnerable and susceptible to environmental toxins. It is also important to note that foods prone to contamination by aflatoxin and fumonisin are also susceptible to other types of mycotoxins, which were not assessed in this study. This shows that these children may be exposed to multiple mycotoxins, which may increase health risks.

Aflatoxin and fumonisin exposure in children was associated with the quantity of maize intake and differed significantly between the different survey periods (at recruitment, at 6 months after recruitment and at 12 months after recruitment) and between the three villages studied. In addition, levels of aflatoxin exposure were found to be higher in children who consumed groundnuts. Maize is highly prone to contamination by both aflatoxins and fumonisins and groundnuts are highly susceptible to contamination with aflatoxins. These

food crops are the potential sources of exposure of children to the mycotoxins. Differences in exposure levels between villages demonstrated the impact of agro-ecological variations in mycotoxins contamination in food and geographical variations in food consumption pattern. Similarly, there was a seasonal variation in magnitude of exposure of children to aflatoxin and fumonisin.

Consumption of dehulled maize and breast feeding seemed to be protective factor for exposure to the two mycotoxins studied. Lower levels of AF-alb and UFB₁ were recorded in children whose complementary foods were based on dehulled maize than in children who consumed whole kernel maize. Food processing methods such as dehulling, sorting to remove defective grains and washing are known to reduce levels of mycotoxin contamination in foods and subsequently reduce exposure of human to the toxins. Levels of exposure to aflatoxin and fumonisin were lower in children who in addition to complementary foods were also breast feeding than in children who had stopped breast feeding. The magnitude of exposure of children to aflatoxins and fumonisins and the key determinants such as age, geographical location, season and child feeding practices have been identified as important factors worth taking into consideration in addressing the problem of mycotoxins exposure in children of Tanzania.

Children showed high prevalence and persistence of under-nutrition during early childhood, with stunting being the most important nutritional problem of public health concern, thus indicating that there is likelihood for lifelong effects due to this chronic nutritional problem. Impaired child growth observed in this study has the potential to be tracked over time, indicating that it was a challenge for malnourished children to recover and improve their nutritional status given the prevailing conditions. Village of residence, age, feeding frequency and breast feeding status were the factors for poor nutritional status.

Stunted growth at early age was a major factor for stunting at later age. In addition, fumonisin exposure and especially when it co-occurs with aflatoxin exposure could be amongst the contributing factors for impaired growth at early childhood in many parts of Tanzania with similar conditions as observed in the present study.

6.2 Recommendations

This study revealed high magnitude of dietary exposure of young children to aflatoxin and fumonisin together with persistently high prevalence of stunted growth and underweight. It is therefore recommended to undertake appropriate measures to address the prevailing situation. While recommending for intervention to minimise human exposure to mycotoxins, it is important to consider that the problem of mycotoxin is basically cross-cutting. In view of this, effective control measures need to be comprehensive and participatory, involving multidisciplinary partners and joint strategies by government, the private sector, farmers, consumers and all other players in the food value chain. In addition, intervention measures to prevent exposure of children to mycotoxins should be considered as one of key initiatives for improving child health and nutrition in Tanzania. The following are specific recommendations suggested for reducing dietary exposure of children to aflatoxins and fumonisins:-

- (i) To raise awareness about the aflatoxin and fumonisin contamination and exposure in humans, the associated health risks and possible measures to reduce contamination and exposure. This should be targeted to policy and decision makers, farmers, agricultural extension workers, food processors, traders, health workers and consumers.
- (ii) To revise national policies, guidelines and strategies, particularly those related to health, nutrition, agriculture, food security and food safety in order to take into account the measures for control of aflatoxins and fumonisins exposure.

- (iii) To incorporate in training curriculum for schools, colleges and universities the issues related to contamination, exposure, health effects and ways to control aflatoxins, fumonisins and other types of mycotoxins. Similarly, this should be incorporated in training curriculum for food processors and farmers.
- (iv) To promote, encourage and support consumption of more varied diet including foods which are less susceptible to mycotoxins contamination.
- (v) To continue promoting and support exclusive breast feeding during the first 6 months of life and appropriate introduction of complementary foods while continuing with breast feeding in order to delay exposure to mycotoxins at early age and to reduce intake of the toxins.
- (vi) To implement measures for preventing exposure of pregnant and breast feeding women to mycotoxins since there is potential for exposure during foetal development and breast feeding as a result of maternal exposure and subsequent transfer of toxic metabolites to unborn and breast feeding child. Such measures include promoting dietary diversification and limit quantities of intake of foods which are susceptible to mycotoxin contamination for pregnant and breast feeding women.
- (vii) To strengthen health and nutrition interventions such as micronutrients supplementation, food fortification, bio-fortification, consumption of foods with antioxidants and immunisation programmes in order to abate the effects of mycotoxins along with improving children nutritional status.
- (viii) To improve post-harvest food practices through proper drying, storage, good manufacturing practices, transportation and food preparation methods such as dehulling, sorting to remove bad quality grains, winnowing, washing as well as other economically feasible methods such as nixtamalisation and fermentation. Measures involving proper drying, storage and food preparation methods are

effective especially in subsistence farming communities where food crops are produced and consumed within households and communities without prior inspection or control.

- (ix) To investigate specific epidemiological circumstances under which aflatoxin or fumonisin may result in growth impairment and other health risks due to their co-exposure.
- (x) Interventions to prevent under-nutrition and improve nutritional status during childhood need to be initiated, focused at very early age and maintained. This study found that majority of children who at beginning of study were stunted or underweight remained under the same status after one year.
- (xi) Intensify monitoring of child growth status and implementation of nutritional strategies for children in communities most at risk of poor child growth since this study revealed a huge inter village variation in prevalence and tracking of stunted growth and underweight.

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APPENDICES

Appendix 1: Informed consent form

Study on dietary exposure to aflatoxin and fumonisin among children below three years in Iringa, Kilimanjaro and Tabora regions, Tanzania

To be completed by the participating child's mother or care provider (please tick the box)

1. I confirm that I have understood the nature of the study
2. I agree for my child to take part in the above study
3. I understand that my child's participation is voluntary and that my child is free to be withdrawn from the study at any time without giving any reason
4. I agree for my child to give blood and urine for research. I understand that giving these samples is voluntary and that I am free to withdraw my approval for use of the sample at any time
5. I agree that the samples and the information gathered from my child can be looked after and stored for use in future studies
6. I understand that research using the samples given may include laboratory tests aimed at understanding the influences of mycotoxin exposures on human health but that the results of these investigations may not have any implications for me personally

For mothers or care provider who can read and write

Name of child

Name of mother or care provider

Signature of mother or care provider

Date

For mothers who can neither read nor write

(To be filled by a village leader/health worker in presence of a mother and a witness)

I witnessed verbal consent given by mother or care provider of the hereunder listed child for her child and herself to participate in the above stated study. It should be understood that the mother/ care provider was informed of the intent of the study, its benefits and the use of the research findings and gave their informed verbal consent willingly.

Name of the child

Name of the mother/ care provider

Thumb mark (in ink)

Name of health worker/village leader

Signature

Date

Name of witness

Signature

Date

Appendix 2: Questionnaire for data collection

Dietary exposure to aflatoxin and fumonisin among children below three years in Iringa, Kilimanjaro and Tabora regions, Tanzania

(To be administered to each mother or care provider of a participating child)

1. General information *(To be filled by the interviewer)*

- (a) Region.....
- (b) Village.....
- (c) Date of visit:.....
- (d) Number of survey (i) At recruitment (ii) At 6 months after recruitment (iii) At 12 months after recruitment
- (e) Name of interviewer.....

2. Child general information

- (a) Child identification number
- (b) Sex (i) Male (ii) Female
- (c) Date of birth.....
- (d) Birth weight.....
- (e) Is the child Immunised against vaccine preventable diseases? *(refer to child's health clinic card)* (i) Yes (ii) No

3. Child feeding information

- (a) Is a child breast feeding? (i) Yes (ii) No
- (b) If no, at what age did the child stop breast feeding?
- (c) At what age was the child introduced to complementary foods

- (d) What type of food do you normally give your child in a week? (i) Plain maize porridge (ii) Mixed flour porridge (iii) Stiff porridge (iv) Rice (v) Cassava (vi) Potatoes (vii) Banana (viii) Others (*mention any other food eaten by the child*).....
- (e) In the last one week, how many days did you give your child maize based meals?
- (f) In the last one week, how many days did you give your child groundnuts based meals?

4. Source of maize and maize processing method

- (a) What is the source of maize used in preparation of child's food?
 (i) Home grown (ii) From the market (iii) Others (*Specify*).....
- (b) Do you process maize used for preparation of child's food? (i) Yes (ii) No
- (c) If yes to 4(b) above, how is the maize processed?
 (i) Dehulled (ii) Washed (iii) Dehulled and soaked (iv) Others (*specify*).....

5. Child's mother or care provider particulars

- (a) What is your age?(years)
- (b) What is your marital status?
 (i) Single (ii) Married/living together (iii) divorced/separated (iv) Widow
- (c) What is your level of education? (i) No formal education (ii) Primary education (iii) Secondary education (iv) Others (*Specify*)
- (d) What is your occupation?
- (e) How many children do you have?
- (f) What is the size of your family?

6. A 24-hour dietary recall for the child

(To be interviewed for two days during each visit)

Time/meals	Types of food	List of all ingredients used in preparation of the food	Quantity of each food type which the child ate and finished (gm or ml)
Morning			
Mid morning			
Lunch			
Mid evening			
Dinner			

Appendix 3: Procedure for collection and handling of urine samples

Urine samples were collected using Hollister U-Bag urine collectors (Hollister Incorporated, 2000 Hollister Drive, Libertyville, Illinois 60048 USA). Each mother or care provider of a participating child was carefully trained and demonstrated on how to collect, handle and protect the urine samples them from light exposure. The following procedures were used:-

- (i) The area around the urethra of the child was thoroughly washed to ensure that the perineal area is clean and dry.
- (ii) The release paper was removed from the adhesive patch of urine bag.
- (iii) Child's legs were separated and the urine bag's narrowest section of the exposed adhesive was placed against the perineum. All the exposed adhesive part of the urine bag was firmly pressed and smoothed against the skin to ensure proper adhesion and avoid wrinkles which can create channels for urine leakage. For males the entire penis was placed in the bag and for females, the bag was placed over the labia. For an active child, support of a second person was required to hold child's legs. A diaper was securely put over the urine bag to avoid displacement of the bag by a child.
- (iv) After the urine was collected, the urine bag was removed by gently peeling of adhesive patch from the skin. The bag was tilted so that the urine is away from the draining tab. The tab was removed and a sample of urine (40 - 50 ml) was carefully drained into a clean 50 ml capacity conical/centrifuge tube with screw lid (SARSTEDT).
- (v) The tube containing urine sample was tightly closed, kept in ice box at study sites until transferred in freezer at -20 °C in within 6 to 8 hours.
- (vi) Frozen urine samples were transported from study areas in ice box and kept frozen under -20 °C in freezer at TFDA laboratory until transported by airway in dry ice to

the laboratory at Leeds Institute of Genetics Health and Therapeutics, University of Leeds in the United Kingdom for analysis.

Appendix 4: Procedure for analysis of aflatoxin albumin adducts by Enzyme Linked Immunosorbent Assay (ELISA)

1.0 Extraction of albumin

1.1 Reagents

- (a) Saturated ammonium sulphate (500 ml water was added to 271 g ammonium sulphate and stirred till nearly dissolved).
- (b) 500 ml 1M acetic acid (471.5 ml distilled water was added to 28.5 ml stock acid).
- (c) Phosphate buffered saline (PBS). The composition for one litre PBS was as follows:-

NaCl	8 g
KCl	0.2 g
KH ₂ PO ₄	0.2 g
Na ₂ HPO ₄ .2H ₂ O	1.15 g

The solution was adjusted to pH 7.4 with HCl (1M) or KOH (1M)

1.2 Controls

Four controls were prepared alongside test samples. The controls contained known concentrations of AF-alb. These were aliquoted and stored in freezer at -20 °C.

1.3 Extraction procedure

- (a) Serum or plasma was placed in a pre-heated water bath (56 °C) for 45 minutes to inactivate any HIV.
- (b) Samples were cooled on ice for five minutes. To 250 µl plasma sample, 375 µl of cold (4 °C) saturated ammonium sulphate was added drop wise.

- (c) The mixture was vortexed and centrifuged (9 000 g, 15 minutes, 0 °C) to remove precipitated immunoglobulins.
- (d) The supernatant containing the albumin was transferred to an eppendorf tube and 50 µl 1M acetic acid was added in order to adjust the solution to pH 5 and precipitate the albumin.
- (e) The mixture was vortexed and centrifuged (9 000 g, 15 minutes, 0 °C) to obtain supernatant (on top) and pellet (at bottom).
- (f) The supernatant was discarded and the pellet was re-dissolved in 500 µl of PBS using wash-board technique and vortexing. The sample obtained from extraction was used for protein quantification and albumin hydrolysis.

2.0 Protein quantification

2.1 Reagents

- (a) BIO-RAD Protein assay dye reagent concentrate (filtered)
- (b) Human Serum Albumin (HSA)

2.2 Procedure

- (a) Sample from albumin extraction were diluted at 1:50 in distilled water (20 µl sample + 980 µl water).
- (b) The 1:50 samples from (a) above were further diluted to 1:10 in distilled water to make a 1:500 dilution.
- (c) A 0.1 mg/ml stock of HSA was prepared in distilled water.
- (d) From the 0.1 mg/ml HSA stock, a series of standards was prepared for use in preparation of the protein standard curve as follows:-

Standard	HSA standard ($\mu\text{g/ml}$)	HSA stock (0.1mg/ml) (μl)	Distilled water (μl)
1	0	0	1000
2	2	20	980
3	5	50	950
4	10	100	900
5	15	150	850
6	20	200	800
7	25	250	750
8	30	300	700

- (e) 40 μl of filtered Bio-rad reagent was pipetted into each well of a 96 well microplate.
- (f) 160 μl of a sample/standard was pipetted in quadruplicate into the wells according to the laboratory protocol and mixed.
- (g) The absorbance was measured immediately at 620 nm in the plate reader to enable calculation of the albumin concentration.

3.0 Albumin hydrolysis

3.1 Reagents

- (a) Pronase [(Pronase solution (10 mg/ml PBS)]: 400 mg pronase + 40 ml PBS
- (b) PBS pH 7.4
- (c) Bovine Serum Albumin
- (d) Absolute ethanol

3.2 Procedure

- (a) A volume of sample (from the extraction procedure 1.0 above), which is equivalent of 2 mg albumin was placed into a 15 ml falcon tube.
- (b) 0.67 mg pronase was added and volume adjusted to 0.8 ml with PBS.
- (c) The mixture was incubated in a water bath at 37 °C for 15 hours.

- (d) 10 mg (100 µl) of bovine serum albumin (100 mg/ml stock solution in PBS) was added.
- (e) Protein was precipitated by adding 1.8 ml cold ethanol.
- (f) Samples were cooled at -20 °C for at least two hours prior to centrifugation (1000 g, 15 minutes, 4 °C).
- (g) Supernatant was diluted to a volume of 30 ml with PBS to reduce the final ethanol concentration to 5% so that the aflatoxin residues could bind to the Sep-pak cartridges during purification.

4.0 Purification

4.1 Reagents

- (a) Methanol [High Performance Liquid Chromatography (HPLC) grade]
- (b) 80% Methanol/water
- (c) 5% Methanol/water
- (d) PBS pH 7.4

4.2 Procedure

All steps were performed using an eight-channel peristaltic pump (Ismatec, supplied by Cole-Parmer) and Sep-pak C-18 cartridges (Waters, MO, USA).

- (a) The lines attached to the peristaltic pump were washed with methanol.
- (b) The cartridges were attached to the pump and washed with 5 ml 80% methanol, followed with 10ml distilled water.
- (c) Hydrolysed sample was loaded in a total volume of 30 ml PBS.
- (d) Cartridges were washed with 5ml distilled water, followed with 5 ml 5% methanol.
- (e) Aflatoxin residues were eluted with 5 ml 80% methanol.

- (f) The eluate was dried in Savant Speed-vac overnight without radiant cover and reconstitute in 0.5 ml PBS for ELISA analysis.

5.0 ELISA

Immunoassay using competitive ELISA, with AFB₁-ovalbumin as the coating antigen and AFB₁-lysine as the inhibitor for the standard curve was used to determine the concentration of AF-alb.

5.1 Reagents

- (a) AFB₁-ovalbumin (for coating ELISA plates)
- (b) AFB₁-lysine inhibitor
- (c) PBS
- (d) PBS Tween 20
- (e) Dried skimmed milk powder (2 g of commercial dried skimmed milk powder was diluted in 40 ml PBS to make 5% milk solution. Fresh solution was made up each day)
- (f) Antiserum, α -ASL8 (primary anti-aflatoxin antibody)
- (g) Foetal calf serum
- (h) Goat anti-rabbit IgG peroxidase labelled antibody (secondary antibody)
- (i) Tetramethylbenzidine (TMB)
- (j) 1M hydrochloric acid (HCl)

5.2 Procedure

- (a) A 96-well ELISA plate (high binding; Greiner bio-one) was coated with AFB₁-ovalbumin. The last row of plate was left as blank.

- (b) The plate was left overnight in drawer (at room temperature) covered with aluminium paper to protect from light.
- (c) Prior to use, the plate was washed 5 times in a wash buffer (PBS + 0.05% Tween 20 solution).
- (d) 200 μ l freshly prepared 5% milk solution in PBS pH 7.4 was added to each well by using a multichannel pipette and incubated at room temperature in dark for 60 minutes.
- (e) The plate was washed five times in a wash buffer (PBS + 0.05% Tween 20 solution).
- (f) Primary anti-aflatoxin antibody was pre-mixed with controls, standards and samples (50:50) as follows:-

PBS: 200 μ l PBS + 200 μ l primary antibody per plate

Samples: 80 μ l sample + 80 μ l primary antibody per plate

Control: 80 μ l control + 80 μ l primary antibody per plate

50 μ l was added to each well using a single channel pipette according to the plate layout. The plate was incubated at room temperature in the dark for 90 minutes on an ELISA plate shaker at 500 rpm.
- (g) Plate was washed five times in a wash buffer (PBS + 0.05% Tween 20 solution).
- (h) 50 μ l of secondary antibody diluted 1:2500 (8 μ l in 20 ml PBS) was added using a multichannel pipette. The plate was incubated at room temperature on the ELISA plate shaker at 500 rpm as before.
- (i) Plate was washed 5 times with PBS Tween 20 followed by one wash in distilled water.
- (j) 50 μ l TMB peroxidase substrate was added per plate well by using a multichannel pipette and plate incubated at 37 °C for 20 minutes.

- (k) 50 μ l of 1M HCl was added by multichannel pipette to terminate the enzymatic reaction.
- (l) The colour absorbance was read at 450 nm in the ELISA plate reader by using standard curve.
- (m) The concentration of AF-alb was calculated from the AFB1-Lysine standard curve.

5.3 Quality control

- (a) ELISA results were accepted when control values lied within the following ranges:-
 - Control 1: 2.0 - 3 fmol/0.1mg albumin
 - Control 2: 1.0 - 1.8 fmol/0.1mg albumin
 - Control 3: 0.7 - 1.4 fmol/0.1mg albumin
 - Control 4: <0.7 fmol/0.1mg albumin
- (b) Each sample was assayed in quadruplicate on at least two separate occasions. Quadruplicate results within each ELISA should have coefficient of variation (CV) below 10%. The inter-day CV for results of each sample tested on separate days should be less than 25%.
- (c) Extractions were repeated on random samples in order to ensure reproducibility of data.

Appendix 5: Procedure for analysis of fumonisin B₁ in urine

The analysis method used Oasis® MAX cartridges for sample clean-up, together with LC-MS method to detect free fumonisin B₁ in urine.

1.1 Reagents

- (a) Fumonisin B₁ standard (concentration: 1000 ng/ml methanol; working solution: 100 ng/ml in methanol:water 1:1)
- (b) FBd6 internal standard (concentration: 1000 ng/ml methanol; working solution: 20 ng/ml in methanol:water 1:1)
- (c) Methanol (HPLC grade)
- (d) Acetonitrile (HPLC grade)
- (e) Formic acid
- (f) Ammonium hydroxide (NH₄OH)
- (g) Mobile phase:- (i) Water:acetonitrile:formic acid (90:10:0.1)
(ii) Water:acetonitrile:formic acid (10:90:0.1)

1.2 Equipments

- (a) Waters Oasis® MAX column (30 µm, 3 cc; 60 mg)
- (b) Waters Alliance HT 2795 separation module HPLC - Waters
- (c) Quattro Micro Mass Spectrometer - Waters
- (d) MassLynx 4.0 operation system with QuanLynx quantification system - Waters
- (e) 4.6 x 150 mm Phenomenex Luna Column (5 µm), protected with a 0.5 µm Phenomenex guard column cartridge

1.3 Procedure for sample preparation

- (a) A urine sample was thawed and centrifuged (5 000 g, 15 minutes, 4 °C).

- (b) 10 ml of the supernatant was taken and mixed with equal volume of double distilled water.
- (c) 80 μ l of internal standard (FBd6 working solution) was added into each sample and into the control samples.

1.4 Extraction procedure

All extraction steps were performed using an eight-channel peristaltic pump (Ismatec, supplied by Cole-Parmer). The FB₁ in urine was isolated by solid-phase extraction (SPE) using a 3CC Oasis^R MAX cartridge (Waters, UK).

- (a) The lines attached to the peristaltic pump were washed with 3 ml of methanol:water (1:1).
- (b) The extraction columns (Oasis[®] MAX) were conditioned with 2 ml of methanol:water (1:1). Care was observed to ensure the columns did not dry out.
- (c) 20 ml of prepared sample and quality control samples were applied to the wet column at flow rate of 0.5 ml/min. All samples were allowed to run evenly and at the same rate.
- (d) The column was washed with 2 ml of 5% ammonium hydroxide solution (5 ml NH₄OH in 100 ml water).
- (e) The column was further washed with 2 ml of methanol.
- (f) The residue was eluted with 2ml of 2% formic acid (2 ml formic acid in 100 ml of methanol) and dried in a Savant Speed-vac without heat for overnight.
- (g) The dried sample was reconstituted in 200 μ l methanol:water (1:1).
- (h) Reconstituted sample was transferred into a 1.5 ml eppendorf tube and centrifuged (5 000 g, 15 minutes, 4 °C).
- (i) The supernatant was carefully removed without disturbing any precipitate, then transfer to the HPLC vial for LC-MS analysis.

1.5 Fumonisin B₁ standard

Five fumonisin FB₁ standards were prepared at 50, 10, 5, 1 and 0 ng/ml with 8ng/ml of FBd6 as internal standard and run with each batch of samples. The standards were prepared as follows:-

Standard	A	B	C	D	E
Concentration FB ₁ (ng/ml)	50	10	5	1	0
Concentration FB ₁ d6 (ng/ml)	8	8	8	8	0
FB ₁ working solution (μl)	1500	300	150	30	0
FBd6 working solution (μl)	1200	1200	1200	1200	0
Methanol:water (1:1) (μl)	300	1500	1650	1770	3000
Total volume (μl)	3000	3000	3000	3000	3000

1.6 Control samples

Blank urine samples was aliquoted at 10 ml volume to serve for positive and negative quality control samples. For positive control, 10 ml of blank urine was spiked with 20 μl of FB₁ working solution. Negative control was a 10 ml blank urine without FB₁ standard.