Human aflatoxin exposure in south-western Ethiopia assessed using serum biomarkers

A thesis submitted in partial fulfilment of the requirements for the Degree of Master of Science

at

Lincoln University

by

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Lincoln University

2020

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Aflatoxins are secondary metabolites produced by the fungi *Aspergillus flavus* and *A. parasiticus.* Consumption of aflatoxin-contaminated food can cause hepatocellular carcinoma, immune suppression, child growth impairment, and death. The study aimed to assess aflatoxin exposure in South-western Ethiopian adults, using serum biomarkers. The study was conducted in five South-western Ethiopian rural districts (Shebe Senbo, Manna, Seka Chekorsa, Kersa, and Omo Nada) in the Oromia Region and included 100 adults aged 18–60 years. The 100 serum samples collected were analysed for aflatoxin B_1 (AFB_1), aflatoxin B_1-lysine (AFB_1-lys), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2) and aflatoxin M_1 (AFM_1) using liquid chromatography-tandem mass spectrometry. All six of the analysed aflatoxins were detected in 64/100 (64%) of the serum samples at levels ranging from 8.3 to 422.9 ng/L. The most frequently detected aflatoxin was AFM_1 (38%), followed by AFB_1 (37%), AFB_1-lys (27%), AFG_1 (25%), AFG_2 (25%), and the least detected was AFB_2 (5%). The mean aflatoxin concentrations were 72.3, 69.9, 58.6, 43.2, 36.9 and 6.1 ng/L for AFB_1-lys, AFM_1, AFG_2, AFB_1, AFG_1 and AFB_2, respectively. There was no association between sociodemographic characteristics and serum aflatoxin concentration. Body mass index (BMI) of the participants did not correlate with their serum aflatoxin concentration. There was a significant association between serum aflatoxin concentration and high millet ($p = 0.031$), groundnut ($p = 0.019$), chickpea ($p = 0.023$), lentil ($p = 0.003$) and soybean ($p = 0.018$) consumption. The results suggest the exposure of South-western Ethiopian adults to aflatoxins. Therefore, rules and regulations on the maximum limits of aflatoxin concentration in food and feed should be implemented by the government. We hope that the results of this study will encourage the implementation of evidence-based interventions and development of food regulations in Ethiopia.

Keywords: Aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, aflatoxin M_1, aflatoxin B_1 lysine, South-western Ethiopia, serum, adult, liquid chromatography-tandem mass spectrometry

Abbreviations

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
AFB_1-lys	Aflatoxin B_1-lysine
BMI	Body mass index
°C	Degrees Celsius
GSH	Glutathione
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
ELISA	Enzyme-linked immunosorbent assays
HPLC	High-performance liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MRL	Maximum residue limit

Acknowledgements

First, I thank God for all the blessings in my life that helped me to finish my study. Learning abroad at a university like Lincoln University, where I could gain better knowledge and experience was my dream. I would like to acknowledge the Ministry of Foreign Affairs and Trade (MFAT) of New Zealand for sponsoring my study and making my dream come true. Sue Bowie and Jayne Borrill, I would have gone back home without completing my study if you had not put all your efforts into solving the problems I faced. Thank you so much for everything. Thanks to the Lincoln University community for creating a positive environment throughout my study.

Due to logistics problems at the study site, I had a lot of disappointing days while conducting my study. But having Professor Ravi Gooneratne as my supervisor helped me out from all those discouraging conditions and I was able to complete my study. Starting from the day I arrived at Lincoln University, I have been requesting his support and he was always there. Thank you, Ravi, for your friendly approach, guiding me throughout my research work, and the time and energy you spent in advising me.

My sincere gratitude goes to Professor Saraha De Saeger for letting my samples be analysed at her laboratory and Drs Marthe De Boevre and Arnau Vidal Corominas for analysing my samples. Thank you to Mytox-South for sponsoring the cost of the aflatoxin analyses.

Special thanks go to all health workers and laboratory technicians at each district health centre in South-western Ethiopia for their friendly approach and collection of samples and data. Thanks go to my friends Mr. Gerba Daba and Dr. Meweal Assefa, for their contribution to data analyses. Thanks also go to my friend Sintayheu for his efforts in following up on the documentation related to the ethical clearance and material transfer agreement at Jimma University, Ethiopia, while I was in New Zealand. My cousin, Eskedar, thank you for your time and energy spent in sending my samples from Ethiopia to Belgium, it was so tiresome.

It is difficult to mention all who supported me during my stay at Lincoln University and my thesis work. Thanks to my family, thank you Alem, and all other friends, for encouraging me. You have no idea what your kind words meant to me.

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Chapter 1
Introduction

Mycotoxins are secondary metabolites of fungi and contaminate soil, decaying vegetation, hay, and different agricultural commodities during the pre- or post-harvest period. In addition to the economic loss caused, mycotoxins impact on human health, the productivity of animals and the food trade; hence they have attracted worldwide attention (Wagacha & Muthomi, 2008). Commonly known mycotoxins are aflatoxins, zearalenone, ochratoxins, deoxynivalenol, fumonisins, trichothecenes, patulin, ergot alkaloids and T-2 toxin (Sherif, Salama, & Abdel-Wahhab, 2009). Aflatoxins are metabolites produced by the fungi *Aspergillus flavus* and *A. parasiticus* and can contaminate food and feed (Gizachew, Szonyi, Tegegne, Hanson, & Grace, 2016). Food products known to have become contaminated by aflatoxins include cassava, chili peppers, cottonseed, millet, peanuts, rice, sesame seeds, sorghum, sunflower seeds, sweet corn, tree nuts, spices and milk (Mohammed et al., 2016; Probst, Bandyopadhyay, & Cotty, 2014). The growth of fungi and associated toxin (fungal metabolite) production is most common in areas with hot and humid climates, especially if the storage conditions of the food/feed is poor (IARC, 2002). During storage, high moisture content of the food/feed and the absence of optimal temperature causes fungal growth, which can lead to the production of aflatoxin (Achaglinkame, Opoku, & Amagloh, 2017). Aflatoxins are grouped as B_1, B_2, G_1, and G_2. After ingestion, aflatoxin B_1 is hydroxylated to aflatoxin M_1 and appears in human and cow milk (Peraica, Richter, & Rašić, 2014).

The International Agency for Research on Cancer has classified “naturally occurring mixes of aflatoxins” as a Group 1 human carcinogen (IARC, 2002). Acute aflatoxin exposure causes aflatoxicosis. One of the most documented aflatoxicosis outbreaks occurred in 2004 in rural Kenya and resulted in 317 cases and 125 deaths (Azziz-Baumgartner et al., 2005; Lewis et al., 2005). Chronic aflatoxin exposure causes hepatocellular carcinoma (Liu & Wu, 2010; Lunn et al., 1997), immune suppression (Jiang et al., 2005; Turner, Moore, Hall, Prentice, & Wild, 2003) and child growth impairment (Gong et al., 2004; McMillan et al., 2018; Turner et al., 2007).

Food analysis and questionnaires are used to assess human aflatoxin exposure. The issue of bioavailability, toxicokinetics, and shortage of data on individual exposure are the limitations of this approach. Therefore, biological methods such as serum aflatoxin biomarkers for

chronic exposure assessment and urinary biomarkers for acute exposure are used to assess aflatoxin exposure at the individual level (Turner, Flannery, Isitt, Ali, & Pestka, 2012).

Studies on the assessment of human exposure to aflatoxin have been conducted in Africa (Dash et al., 2006; Ezekiel et al., 2014; Yard et al., 2013), South America (Jager, Tonin, Baptista, Souto, & Oliveira, 2016; Jager, Tonin, Souto, Privatti, & Oliveira, 2014) and Asia (Ali et al., 2016; Tao et al., 2005). The aflatoxin concentration in the serum or urine of humans may be affected by different factors, like the place of residence, gender, age, food frequency data, BMI, or occupation. In Bangladesh, a significantly higher level of AFM_1 was found in a rural cohort (99 ± 71 pg/mL) than an urban one (54 ± 15 pg/mL) and the concentration of urinary AFM_1 was positively correlated with rice consumption (Ali et al., 2016). A study from Uganda revealed that the type of residential area and occupations had a significant difference in the serum AFB_1-lys adducts (Kang et al., 2015). A survey from the Portuguese waste industry showed that serum AFB_1 levels ranging from 2.5 to 25.9 ng ml^{-1} were detected in all waste workers, compared to the controls, whose levels were below the detection limit (1 ng ml^{-1}) (Viegas et al., 2014).

In Ethiopia, studies have reported the occurrence of aflatoxins in various food commodities including cereals (Ayalew, Fehrmann, Lepschy, Beck, & Abate, 2006), groundnut (Chala, Mohammed, Ayalew, & Skinnes, 2013; Mohammed et al., 2016), milk (Gizachew et al., 2016) and beer (Nigussie, Bekele, Gemede, & Woldegiorgis, 2018). To my knowledge, no studies have reported on the assessment of human aflatoxin exposure using serum and urine biomarkers, except for one report. Ayelign et al. (2017) detected urine aflatoxins in 1–4-year-old children from the Tigray and Amhara regions of Ethiopia. The AFM_1 contamination was 7% (14/200) with values ranging from 0.063 to 0.070 ng/mL, AFB_2 = 4.5% (9/200; < limit of quantification (LOQ) to 0.063 ng/mL), AFG_2 = 3% (6/200; 0.066 – 0.070 ng/mL) and AFG_1 = 2.5% (5/200; 0.054 – 0.065 ng/mL).

1.1. Statement of problem

In addition to the food shortage, the safety of food is a priority issue for developing countries. On the African continent, for example, mycotoxin contamination is a significant food safety issue. Poor production and storage conditions of African primary staple foods lead to the development of moulds that produce aflatoxins (Lewis et al., 2005). The consumption of aflatoxin-contaminated foods, inhalation, and dermal contact with aflatoxins cause

aflatoxicosis, hepatocellular carcinoma, child growth impairment and immune suppression. In African countries, it is essential that evidence-based interventions are implemented that fit the African context and those mandatory regulations are developed based on local conditions. To provide the needed evidence, food analysis, questionnaires and biological markers can be used to assess the exposure of humans to aflatoxin. The latter is preferable as it helps to measure aflatoxin exposure from all exposure pathways. Food analysis reflects only the amounts ingested via the oral route. Therefore, this study was conducted to evaluate the occurrence and concentration of AFB_1, AFB_2, AFM_1, AFG_1, AFG_2, and AFB_1-lys in the serum of South-western Ethiopian adults. We hope the results of this study will help in the implementation of evidence-based interventions and regulations in Ethiopia.

1.2. Aims and objectives

The study aimed to assess aflatoxin levels in the serum of South-western Ethiopian adults in relation to diet and demographic factors.

The objectives of this research were to:

1. Investigate the occurrence of aflatoxins (AFB_1, AFB_2, AFM_1, AFG_1, AFG_2, and AFB_1-lys) in the serum of adults living in South-western Ethiopia.
2. Measure the concentration of aflatoxins (AFB_1, AFB_2, AFM_1, AFG_1, AFG_2, and AFB_1-lys) in the serum of these adults.
3. Evaluate the association between serum aflatoxin concentration and food frequency data.
4. Assess the association between serum aflatoxin concentration and sociodemographic characteristics.
5. Evaluate whether there is a correlation between serum aflatoxin concentration and body mass index (BMI).

1.3. Research questions

The research questions addressed in this study were:

1. Do aflatoxins exist in the serum of adults living in South-western Ethiopia?

2. If aflatoxins exist in the serum of these adults, what are the concentrations?

3. Do the types of foods and frequency consumed by the study population influence serum aflatoxin concentration?

4. Is there any association between serum aflatoxin concentration and sociodemographic characteristics?

5. Is there any correlation between serum aflatoxin concentration and BMI?

1.4. Research hypotheses

i. The serum aflatoxin concentration will be relatively high in adults living in South-western Ethiopia.
ii. There will be an association between serum aflatoxin concentration and sociodemographic characteristics and the type and frequency of food consumed by the study population.
iii. Serum aflatoxin concentrations will vary with the participants' BMI.

1.5. Structure of thesis

This thesis comprises six chapters: 1. Introduction, 2. Literature Review, 3. Materials and Methods, 4. Results, 5. Discussion, and 6. Conclusions.

Chapter 1 gives a general introduction to the study and states the problem, aims, objectives, research questions, and research hypothesis.

Chapter 2 comprises a detailed literature review on aflatoxins, their occurrence in agricultural commodities in Africa and particularly in Ethiopia, and factors affecting the occurrence of aflatoxigenic fungi and aflatoxins. The review also covers the different exposures of humans to aflatoxin and their impact on human health, the methods used to detect different groups of aflatoxins, and methods to prevent and control exposure to aflatoxins.

Chapter 3 describes the methods followed during data and sample collection and gives general information on the study area and the study population. It also describes the procedure used for aflatoxin analysis and the materials, chemicals and reagents used for sample collection and laboratory analysis.

Chapter 4 presents the results, which consist of serum aflatoxin concentration and its relationship with sociodemographic characteristics and the food-frequency of the participants. The relationship between serum aflatoxin concentration and BMI is also presented. The results are presented in the form of graphs, tables, and text.

Chapter 5 discusses the results of the current study and compares them with the results of studies reported in the literature.

Chapter 6 summarises the results of the research and provides a general conclusion. It also discusses the limitations of this study and makes a recommendation on how future researchers could use the results of this study to fill knowledge gaps.

Chapter 2

Literature Review

2.1. Occurrence of aflatoxins in African foods

The aflatoxin concentration in most foods commonly consumed in Africa is above the maximum residue limit (MRL) recommended by the EU (Alshannaq & Yu, 2017) and hence cause health problems in the population. For example, in the city of Lilongwe, Malawi, 67 groundnut products (raw shelled groundnuts, groundnut flour and groundnut butter) were purchased from supermarkets and vendors and analysed for aflatoxins. Analysis of the samples revealed that total aflatoxin concentrations in raw peanuts and groundnut flour purchased from vendors ranged from 1.5 to 1200 µg/kg and from 83 to 820 µg/kg, respectively. Aflatoxin concentrations in supermarket samples ranged from 13 to 670 µg/kg for groundnut flour and 1.3 to 180 µg/kg for groundnut butter. Aflatoxin concentrations in 93% of the samples were above 3 µg/kg (maximum permissible limit in Malawi) (Magamba et al., 2017).

In the Democratic Republic of Congo and Burundi, analysis of 244 samples of milk, groundnut, soybean, beans, sorghum, maize, cassava and their processed products collected from the local markets revealed aflatoxin in all samples at concentrations ranging from 1.3 to 2,410 µg/kg. The average aflatoxin concentration of the samples from Burundi was higher (99.6 µg/kg) than that (29.3 µg/kg) in the Democratic Republic of Congo samples (Udomkun et al., 2018). The highest aflatoxin concentration was found in processed products of sorghum, maize, and groundnut, compared with that in unprocessed grain, which shows that aflatoxin is not removed by processing. The AFM_1 concentration in milk and dairy products ranged from 4.8 to 261.1 ng/kg. The total aflatoxin concentration in 51% of the crop samples was above 4 µg/kg (the EU MRL). In another study from The Democratic Republic of Congo (Kamika & Takoy, 2011), in 60 raw peanut samples collected from rural parts of Kinshasa during both rainy and dry seasons, AFB_1 was found in 90% of peanut samples collected during the rainy season at concentrations ranging from 12 to 937 µg/kg, whereas, in peanuts collected during the dry season, AFB_1 was found in 53% of samples with concentrations ranging from 1.5 to 390 µg/kg. In both seasons, the AFB_1 concentration in 90% of the peanut samples was above the MRL of AFB_1 recommended by the EU (2 µg /kg).

In Nigeria, samples of the food products ‘egusi’ (melon seeds), ‘robo’ (melon ball snacks), ‘ogiri’ (fermented melon seed condiment) and ‘egusi’ soup were collected from households and restaurants in 2005 and 2006 for aflatoxin analysis. Results showed that 25% of ‘robo’, 31.8% of ‘ogiri’ and 19.5% of ‘egusi’ soup were contaminated with AFB_1 with mean concentrations of 9.7, 8.9 and 7.2 ppb, respectively. The aflatoxin concentration in all samples ranged from 2.3 to 15.4 ppb, which is lower than Nigeria’s MRL of 20 ppb (Bankole, Adenusi, Lawal, & Adesanya, 2010). The authors concluded that the aflatoxin concentration contribution of all daily food consumed should be considered in evaluating overall exposure. In another study conducted in Nigeria, on 80 meat samples collected from Oyo state, major aflatoxins (B_1, B_2, G_1, and G_2) were detected in all samples. The samples included fresh and sun-dried beef, liver, kidney, and heart. In the fresh samples, the total AFB (AFB_1 plus AFB_2) concentration ranged from 0.0217 to 0.0852 μg/kg and total AFG (AFG_1 plus AFG_2) from 0.0267 to 0.0741 μg/kg. In the dried samples, total aflatoxin concentrations were lower and ranged from 0.0029 to 0.0758 μg/kg for AFB and 0.0031 to 0.1413 μg/kg for AFG (Oyero & Oyefolu, 2010). These results indicated that the animals had consumed aflatoxin-contaminated feed. In research that compared aflatoxin contamination in dried red chillies from markets in Nigeria with those purchased in the US, a significantly higher concentration of AFB_1 was detected in Nigerian chillies (13.5 μg/kg) compared to in the ones from the United States (5.1 μg/kg) (Singh & Cotty, 2017).

In Zambia, a 3-year comprehensive aflatoxin analysis of peanut butter revealed regular aflatoxin contamination. In that study (Njoroge et al., 2016), of 24 local and imported peanut butter brands, a total of 954 containers were collected from a shop, and 73%, 80% and 53% of the samples tested in 2012, 2013 and 2014, respectively, were positive for AFB_1. As the imported brands tested were from South Africa, Zimbabwe and Malawi, the results suggested contamination of peanuts in these countries also. In a survey conducted in the Shamva and Makoni districts of Zimbabwe, AFB_1 was detected in maize samples. A total of 388 samples (75 maize grain and 313 maize meal) were collected from the two districts. The results revealed AFB_1 occurrence in 80 samples with a range of 0.57 to 26.6 μg/kg. Of the contaminated samples, AFB_1 in 18 samples was above 5 μg/kg (maximum permissible limit in Zimbabwe) (Murashiki et al., 2017).

In Libya, in research to assess AFM_1 concentration in raw cow milk and fresh white soft cheese samples collected from the north-west provinces, AFM_1 was detected in 71.4% of

milk samples and 75% of white soft cheese samples and the concentrations ranged from 0.03 to 3.13 ng/ml and 0.11 to 0.52 ng/g, respectively (Elgerbi, Aidoo, Candlish, & Tester, 2004).

Aflatoxin contamination was also found in spices in West Africa. According to Akpo-Djènontin, Gbaguidi, Soumanou, and Anihouvi (2018), dried spices and aromatic herbal powder collected from Benin were contaminated by AFB_1, AFG_1, and AFG_2. In that study, 66 samples of dried spices and aromatic herbs were collected from processing sites, supermarkets and markets and found to be contaminated with AFB_1 (84%), AFG_1 (32%) and AFG_2 (28%) at concentrations ranging from 0.46 to 84.84 µg/kg, 0.24 to 8.56 µg/kg and 0.11 to 3.68 µg/kg, respectively. All brands of complementary foods (cereal-legume blends and cereal only) collected from all regions of Ghana were found to be affected by aflatoxin contamination. The concentration of aflatoxin ranged from 1 to 11.7 ppb for cereal-only samples and 1 to 1,094 ppb for cereal-legume blends. The highest aflatoxin concentration was detected from the Central region and the lowest from the Upper East region (Opoku, Achaglinkame, & Amagloh, 2018). The research of Lahouar, Jedidi, Sanchis, and Saïd (2018) revealed the contamination of sorghum samples by AFB_1. In the study, out of 64 Tunisian and Egyptian sorghum samples collected from the Tunisian markets, AFB_1 ranged from 0.03 to 31.7 µg/kg and was detected in 59.4% of the samples. Thus, the contamination of different food items by aflatoxin has been reported in almost every region of the African continent.

2.1.1. Occurrence of aflatoxins in Ethiopian foods

Unlike other African countries, only a few studies have been conducted on aflatoxin and aflatoxigenic fungal contamination of Ethiopian foods. Out of these, the report by Chala et al. (2013) revealed the contamination of groundnut samples collected from important groundnut production districts of eastern Ethiopia. In the study, 93 of 120 groundnut samples were contaminated by aflatoxins at concentrations ranging from 15 to 11,900 µg/kg. Another study in the same districts reported by Mohammed et al. (2016) confirmed the high aflatoxin contamination in groundnut seeds and cake. In that study, 160 groundnut seed samples and 50 groundnut cake "Halawa" samples were collected during the cropping seasons of 2013/14 and 2014/15. Aflatoxins (B_1, B_2, G_1, and G_2) ranging from 0.1 to 2526 ng/g were detected in the samples. These studies indicate contamination of groundnut and its products in eastern Ethiopia.

Out of 15 domestic and three imported brands of beer samples collected from Addis Ababa, Ethiopia, 11 of 12 domestic alcoholic beer brands were contaminated by total aflatoxins ranging from 1.23 to 12.47 μg/L. In contrast, the aflatoxin concentrations of non-alcoholic domestic beer brands and imported beer brands were below the limit of detection and limit of quantification, respectively. It is a mandatory to set rules and regulations regarding aflatoxin concentration in beer and other food commodities in Ethiopia (Nigussie, Bekele, Gemede, et al., 2018). In another study from Addis Ababa, all 110 milk samples collected from dairy farmers and milk traders and 156 feed samples from dairy farmers, feed producers, traders, and processors were contaminated with AFM_1 and AFB_1 (Gizachew et al., 2016). AFM_1 ranged from 0.028 to 4.98 μg/L and AFB_1 from 7 to 419 μg/kg in milk and feed samples, respectively.

In a study conducted to assess the occurrence of different mycotoxin types in barley, sorghum, teff and wheat from Ethiopia, AFB_1 was detected in all cereal samples. AFB_1 concentration ranged from < 1 to 26 μg/kg in 8.8% of the 352 samples. The maximum concentration (26 μg/kg) was detected in sorghum; the reason for this could be the underground storage of sorghum, which leads to a high seed moisture content (Ayalew et al., 2006). In another study comparing aflatoxin concentrations in fresh and stored sorghum samples, a higher concentration of AFB_1 was detected in stored than in fresh sorghum samples. The aflatoxin analysis of sorghum samples collected from eastern Ethiopia revealed a lower concentration of AFB_1 (17 μg/kg) in fresh sorghum than in samples stored for 5–6 months (33.1 μg/kg) (Taye, Ayalew, Chala, & Dejene, 2016). A study by (Chala et al., 2014) also confirmed the detection of AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1 in sorghum samples and AFB_1 and AFG_1 in finger millet samples. These findings suggest the wide aflatoxin contamination of different Ethiopian foods and hence an urgent implementation of rules and regulations regarding aflatoxin concentration is required to prevent further health issues related to aflatoxin toxicity.

Table 1.Aflatoxin contamination of foods from different African countries

Country	Year of study	Food commodity (product)	Source of the commodity	Number of samples	Aflatoxin type	Positive samples (%)	Mean (µg/kg)/ *Median	Range (µg/kg)	Reference
Malawi	2017	Raw shelled peanut	Vendors	67	Total AF	100	182	1.5 to 1200	Magamba et al. (2017)
		Groundnut flour					187	83 to 820	
		Groundnut flour	Supermarkets				279	13 to 670	
		Groundnut butter					77	1.3 to 180	
Democratic Republic of Congo	2016	Milk Groundnut Soybean Beans Sorghum Maize Cassava	Local markets	244	Total AF	100	29.3	1.3 to 2,410	Udomkun et al. (2018)
Burundi									
Democratic republic of Congo	2009	Raw peanuts	Street hawkers Local markets Retail shop	60	AFB_1	72	99.6 -	1.5 to 937	Kamika and Takoy (2011)
Nigeria	2005	'robo' (melon	House hold	233	AFB_1	32.5	9.7	2.3 to 15.4	Bankole et al. (2010)

	and 2006	ball snacks)	Restaurants						
		‘ogiri’ (fermented melon seed condiment)				25	8.9		
		‘egusi’ soup				17.5	7.2		
Nigeria	-	Dry meat	Markets	80	AFB	100		0.0029 to 0.0758	Oyero and Oyefolu (2010)
					AFG			0.0031 to 0.1413	
		Fresh meat			AFB			0.0217 to 0.0852	
					AFG			0.0267 to 0.0741	
Nigeria	2015-2016	Chillies	Markets	55	AFB_1	93	13.5	ND-156	Singh and Cotty (2017)
Zambia		Peanut butter	Shops	954	AB_1				Njoroge et al. (2016)
	2012	Imported brands				-	10	1 to 74	
		Local brands					24	1 to 263	
	2013	Imported brands					55	1 to 10,740	
		Local brands					130	1 to 4,375	
Zimbabwe	-	Maize	Households	388	AFB_1	20.6	*3.21	0.57 to 26.6	Murashiki et al. (2017)

Libya	2002	Raw cow milk	Local dairy factories	49	AFM_1	71.4	-	***0.03 to 3.13	Elgerbi et al. (2004)
		Fresh white soft cheese		20		75		****0.11 to 0.52	
Benin	-	Dried spices and aromatic herbal powder	Processing sites	66	AFB_1	84		0.46 to 84.84	Akpo- Djènontin et al. (2018)
			Supermarkets		AFG_1	32		0.24 to 8.56	
			Markets		AFG_2	28		0.11 to 3.68	
Ghana	-	Cereal-legume blends	Supermarkets	42	Total AF	-	-	1 to 1,094	Opoku et al. (2018)
			Mini-marts						
		Cereal only		6				1 to 11.7	
Tunisia	2011-2012	Sorghum	Market	64	AFB_1	59.4	1.71	0.03 to 31.7	Lahouar et al. (2018)
Ethiopia	-	Groundnut	Farmers' stores	120	Total AF	77.5	-	15 to 11,900	Chala et al. (2013)
			Markets						
	2013-2015	Groundnut seed samples and	Farmers' stores	160	Total AF	-		****0.1 to 2526	Mohammed et al. (2016)
		Groundnut cake "Halawa"	Cafe Restaurants	50					

2015/16	Domestic beer brands	Markets	15	Total	73.3		**1.23 to 12.47	Nigussie, Bekele, Gemede, et al. (2018)
	Imported beer brands		3		-	-	-	
2014/15	Milk	Dairy farmers Milk traders	110	AFM_1	100	-	**0.028 to 4.98	Gizachew et al. (2016)
1999	Barely Sorghum Teff Wheat	Threshing yards Traditional storage structures	352	AFB_1	8.8	5.9	<1 to 26	Ayalew et al. (2006)
2013	Sorghum	Farmers' threshing floors Underground pits	90	AFB_1	-	17 33.1	0.01 to 33.1	Taye et al. (2016)

*= Median; **= µg/L; ***= ng/ml; ****= ng/g; ND=Not detected

2.2. Factors associated with the occurrence of aflatoxigenic fungi and aflatoxins in foods

2.2.1. Agro-ecology and cultivar

The contamination of crops by aflatoxin can be affected by a variety of factors, including agroecology, pre- and post-harvest conditions, and crop cultivar. Mutegi, Ngugi, Hendriks, and Jones (2009) showed an association between aflatoxin concentration and agroecological zone and crop cultivar. In that study, out of the total 769 groundnut samples monitored, 87% contained <4 μg/kg of aflatoxins, 5% of the samples ≥4 μg/kg, and 8% >20 μg/kg (maximum permissible limit in Kenya). There was a significant association between agroecological zone and aflatoxin concentration, with 10% of the samples from the wetter and humid zone containing an aflatoxin concentration of >20 μg/kg and only 3% showing such high values from the dry zone. The association between improved cultivar and aflatoxin concentration is highly significant with improved cultivars reducing the risk of contamination to half. In the same study area, AFB_1, AFB_2, AFG_1, and AFG_2 production was assessed by isolating 1458 cultures of *Aspergillus flavus* or *A. parasiticus* from selected samples. The incidence of *A. flavus* L-strain was highest (78%) > *A. flavus* S-strain (68%) > *A. niger* (65%). The probability of containing total aflatoxin exceeding 10 μg/kg was three times higher in samples from the Busia districts than from the Homa Bay districts. There was a negative correlation between the incidence of AFB and crop rotation, between *A. flavus* and grading, being a member of a producer marketing group, and planting improved cultivars. Therefore, it was reported that the modification of these factors might reduce the hazard of aflatoxin contamination in groundnut (Mutegi, Ngugi, Hendriks, & Jones, 2012). In another study, by Bumbangi et al. (2016), 92 groundnut samples were collected from open markets and supermarkets of Zambia for aflatoxin analysis. Aflatoxin concentrations ranged from 0.014 to 48.67 ppb in 55% of the samples. The variety of peanut was significantly associated with the aflatoxin concentration and out of the three varieties studied (Chalimbana, Kadononga and Makulu red), Chalimbana was considered the riskiest variety and was 3.58 times higher for contamination by aflatoxin than Kadononga and Makulu red.

2.2.2. Storage

Kachapulula, Akello, Bandyopadhyay, and Cotty (2017) revealed an association of agroecology, fungal morphology and storage conditions with the concentration of aflatoxin contamination in maize and groundnut. Out of the 334 groundnut and maize samples collected from 27 districts of Zambia, aflatoxin concentrations > 10 µg/kg (maximum permissible limit in Zambia) were found in 17% of the samples. Out of the samples with aflatoxin concentration ≥ 10 µg/kg, 38% were from Agroecology I, which is the warmest region, and 8% from Agroecology III, the coolest. A lower concentration of aflatoxin was found in maize (16 µg/kg) than in the groundnut (39 µg/kg). There was a 1,000-fold increase in safe crops that had been stored under poor storage conditions at temperatures > 31°C and relative humidity of 100% for a week. For *Aspergillus flavus* L-morphotype, an increase in aflatoxin concentration and time after the harvest of groundnut were negatively correlated. During the marketing of groundnut seeds, different risk factors can increase aflatoxin concentration. Manizan et al. (2018) reported storage of peanuts for 3 months could cause market loss due to fungi (37%) and high temperature and humidity (4.5%). The incidence of those fungi leads to the occurrence of mycotoxins, including aflatoxin.

2.2.3. Threshing

The threshing method is one of the factors associated with aflatoxin contamination. For example, from the research done by (Taye, Ayalew, Dejene, & Chala, 2018), sorghum grain samples threshed on bare ground, canvas, concrete asphalt, and cow-dung-painted ground have different concentrations of aflatoxin. The results showed that *Aspergillus* species were found in all samples from different threshing methods but were higher in bare-ground-threshed samples. As the storage time increased, there was a gradual increase in the aflatoxin B_1 concentration: the highest was in bare-ground-threshed samples (1.97 µg/kg) and the lowest in canvas-threshed samples (0.70 µg/kg).

2.3. Health impact of aflatoxin exposure

2.3.1. Acute aflatoxicosis

Acute aflatoxicosis in humans has been reported in many countries, including developing countries. Vomiting, abdominal pain, pulmonary oedema and fatty infiltration and necrosis of the liver were the clinical manifestations of aflatoxicosis (Wu, Groopman, & Pestka, 2014). One of the largest documented aflatoxicosis outbreaks occurred in 2004, with 317 cases and 125 deaths in rural Kenya. The major source of the outbreak was aflatoxin-contaminated homegrown maize. In a survey conducted on 65 markets and 243 vendors, 350 maize products were collected from the most affected districts, with maize aflatoxin concentration > 20 ppb (maximum permissible limit in Kenya) detected in 55% of samples, > 100 ppb in 35% of samples and > 1,000 ppb in 7% of samples. Higher market maize aflatoxin concentrations were detected in the Makueni district, which had the most aflatoxicosis cases (geometric mean aflatoxin concentration = 52.91 ppb) than in the Thika district, where the concentrations were relatively low (7.52 ppb). Compared to maize bought from other regions of Kenya or other countries, maize collected from farmers of the affected area was significantly more likely to have aflatoxin concentrations > 20 ppb [OR = 2.71; (1.12–6.59)]. In this outbreak, the exposure of individuals to aflatoxin was confirmed by measuring blood biomarker, aflatoxin-albumin adducts (Azziz-Baumgartner et al., 2005; Lewis et al., 2005; Probst, Njapau, & Cotty, 2007; Strosnider et al., 2006). Probst, Callicott, and Cotty (2012) reported that deadly strains of Kenyan *Aspergillus* are distinct from other aflatoxin-producing fungi and this may have been the cause of the largest outbreak of aflatoxicosis in Kenya.

2.3.2. Child growth impairment

Aflatoxin exposure has been linked with childhood stunting. Stunting is a condition in which the height of the child for his/her age is at least two standard deviations below the growth reference of WHO for a country/region. From a public health perspective, stunting is important because of its association with effects like vulnerability to infectious diseases and cognitive impairment (Wu et al., 2014). Though the mechanism of the association between aflatoxin exposure and child impairment is not clearly understood, the possibility of

compromised intestinal integrity, endothelial cell toxicity, which alters barrier function, and immune suppression are valid hypotheses (Wild & Gong, 2009).

Different studies have been conducted to confirm the association between aflatoxin exposure and child growth impairment with the aflatoxin exposure commencing during pregnancy. For example, Turner et al. (2007) revealed the effect of maternal aflatoxin exposure on the growth of Gambian infants. A decrease in the aflatoxin-albumin concentration in the maternal blood (110 to 10 pg/mg) leads to a gain in weight (0.8 kg) and height (2 cm) of infants in their first year of life. Shuaib et al. (2010) also suggested an inverse association between maternal aflatoxin exposure and birthweight of babies. In the study, 785 Ghanaian pregnant women had AFB_1-lys ranging from 0.44 to 268.73 pg/mg albumin. The probability of having a low birthweight baby is higher in mothers with very high AFB_1-lys concentrations (>11.34 pg/mg). It has been shown that an increase in the aflatoxin concentration in children is associated with the reduction in height gain. In a longitudinal study on Benin children aged from 16 to 37 months, an association between the aflatoxin-albumin concentration and growth rate was found. After an 8-month follow-up period, a mean 1.7 cm growth reduction was observed in the high aflatoxin exposure group (Gong et al., 2004). McMillan et al. (2018) also reported an association between AFB_1-lys concentration and stunting, severe acute malnutrition and kwashiorkor in Nigerian children 6–48 months of age. AFB_1-lys concentration ranged from 0.2 to 59.1 pg/mg albumin in the study population. A high concentration of AFB_1-lys was reported in stunted (median = 4.6 pg/mg), severely malnourished (4.3 pg/mg) and children with kwashiorkor (6.3 pg/mg).

Dietary exposure is also used to measure the association between child growth and aflatoxin. Weaning flour samples from 242 households were collected from the Kisumu district of Kenya. Weaning flour is used to prepare the complementary food for infants after 6 months, as a supplement to breast milk. The weight and height of children from each household were measured to assess the children's nutritional status. Based on the results, 31% of the children were malnourished and 29% of the samples positive for aflatoxin. The association between the number of 'wasted' children and being fed aflatoxin-contaminated flour was highly significant. Aflatoxin concentration ranged from 2 to 82 µg/kg in the weaning flour samples (Okoth & Ohingo, 2004).

There is a contradiction between the results of two studies on the correlation between aflatoxin exposure and micronutrient deficiency. Tang et al. (2009) showed a negative

correlation between aflatoxin-albumin adducts and vitamin A and E concentrations in Ghanaian adults aged 18–85 years. The average concentration of serum aflatoxin-albumin adducts of the study population was 0.94 ± 0.64 pmol/mg albumin, vitamin A = 1.32 ± 0.48 µmol/L and vitamin E = 15.68 ± 4.12 µmol/L. Serum aflatoxin-albumin adducts had a significant negative correlation and a strong negative correlation with vitamin A and E concentrations, respectively. In contrast, the study from Benin failed to show a relationship between aflatoxin-albumin concentration and plasma vitamin A and zinc (Gong et al., 2004).

2.3.3. Hepatocellular carcinoma

Aflatoxin exposure causes liver cancer in humans and a variety of animal species. The International Agency for Research on Cancer has classified "naturally occurring mixes of aflatoxins" as a Group 1 human carcinogen (IARC, 2002). In developing countries, the risk of hepatocellular carcinoma is significantly increased if the person is exposed to aflatoxins and the hepatitis B virus (HBV) (Unnevehr & Grace, 2013). Individuals exposed to both aflatoxins and HBV have a greater risk of developing hepatocellular carcinoma than those exposed to only aflatoxin (Wu et al., 2014). There is a variation in the incidence of hepatocellular carcinoma in the world and the highest burden is in Asia and Sub-Saharan Africa (Liu & Wu, 2010). In China, there are >750,000 new cases each year and the annual death rate is >300,000 (Wu et al., 2014).

The association between aflatoxin exposure and human hepatocellular carcinoma has been studied via cross-sectional, ecological, case-control and prospective cohort investigations in highly exposed populations (Wu et al., 2014). Van Rensburg et al. (1985) suggested, in Mozambique, a dose-dependent increase in liver disease associated with an increase in aflatoxin intake, and the mean dietary aflatoxin intake was positively correlated with the hepatocellular carcinoma rates. In Guangxi Zhuang, China, people living in areas with low aflatoxin contamination had a 10-fold lower hepatocellular carcinoma incidence than individuals whose sera were positive for the hepatitis B virus surface antigen (HBsAg+) and who experienced heavy aflatoxin exposure. People who were HBsAg+ and consuming diets with low aflatoxin concentration had a lower rate of hepatocellular carcinoma than those with HBsAg- and who were heavily exposed to aflatoxin (Yeh et al., 1989). In Taiwan, a case-control study was conducted by measuring blood biomarkers of aflatoxin, aflatoxin-albumin adducts and aflatoxin-DNA adducts. In comparison to matched controls, the proportion of

subjects with detectable concentrations of blood aflatoxin-albumin adducts was higher for those that had hepatocellular carcinoma [odds ratio (OR) 1.5]. The presence of detectable blood aflatoxin-albumin adducts and hepatocellular carcinoma among men younger than 52 years were significantly different (multivariate-adjusted OR 5.3) (Lunn et al., 1997). A study from China by Sun et al. (1999) suggested that aflatoxin exposure detected by the presence of AFM_1 in urine is related, in large part, to the risk of hepatocellular carcinoma in men with chronic hepatitis B virus (HBV). In a study conducted to determine whether simultaneous exposure to hepatitis C virus (HCV), exposure to aflatoxin, or family history of hepatocellular carcinoma increased the risk of developing hepatocellular carcinoma, 145 men with chronic HBV were examined. Before the beginning of the follow-up, 8-monthly urine samples were collected to analyse for AFM_1. In 78 (54%) of the subjects, AFM_1 was detected and the increase in the risk of hepatocellular carcinoma was 3.3-fold (95% confidence interval of 1.2–8.7) in those with urinary AFM_1 > 3.6 ng/L. The attributable risk from aflatoxin exposure as the occurrence of detectable AFM_1 was 0.553 (0.087, 0.94). The relative risk of fatal cirrhosis for individuals with elevated urine AFM_1 was 2.8 (0.6, 1.43) (Sun et al., 1999). In another study, hepatocellular carcinoma cases and controls were nested within a cohort and found that in HBV-infected people, there was an adjusted odd ratio (OR) of 2.8 for detectable blood aflatoxin-albumin adducts compared to non-detectable and 5.5 for high concentrations of aflatoxin metabolites in urine compared to those with lower concentrations (Wang et al., 1996).

The target organ for the metabolism of AFB_1 is the liver. After consumption of aflatoxin-contaminated food, AFB_1 is metabolised by cytochrome-P450 enzymes to reactive genotoxic intermediates (aflatoxin B_1-8, 9-oxide, AFBO) or hydroxylated (to AFQ_1 and AFM_1) and demethylated (to AFP_1) to become less harmful than AFB_1. To exert its hepatocarcinogenic effect, AFB_1 must be bio-transformed by the cytochrome-P450 enzyme, which results in the production of a reactive intermediate chemical compound, AFBO. This highly reactive genotoxic compound binds to liver cell DNA as a result, and DNA adducts are formed, namely 8, 9-dihydro-8 (N7guanyl) -9-hydroxy-AFB_1 (AFB_1 N7-Gua). If this is not repaired before DNA replication, the DNA adducts interact with the guanine base of the DNA and cause mutational effects in the p53 tumour suppressor gene, resulting in hepatocarcinogenesis (Hamid, Tesfamariam, Zhang, & Zhang, 2013). Therefore, inhibition of this route using different natural chemicals is used as an intervention method to prevent hepatocellular carcinoma (Figure 1).

The increase in aflatoxin-DNA adduct formation in the liver is proportional to the aflatoxin exposure dose (Buss, Caviezel, & Lutz, 1990). In this study, three exposure concentrations of AFB_1 (0.02, 0.06 and 20 g/L) were given to male F-344 rats in drinking water. Adduct concentrations of 0.91, 32 and 850 nucleotide-aflatoxin per 109 nucleotides were detected in the liver at 8 weeks. In another study, Fisher-344 male rats were fed AFB_1 at concentrations of 0.01, 0.04, 0.4 and 1.6 ppm for 8, 12, 16 and 20 weeks. The results showed that when compared to rats euthanised immediately after the last dosing cycle (12 and 20 weeks of intermittent exposure), a 2-fold decrease in DNA adducts in the liver was observed in rats removed from the treatment 1 month after 1 or 2 dosing cycles (8 and 16 weeks of intermittent exposure). Thus, from exposure to 0.01 ppm to 1.6 ppm of AFB_1, at 12 and 20 weeks of intermittent treatment, there was an increase in liver DNA and RNA adducts (Sotomayor et al., 2003). A rat study revealed that exposure to AFB_1 can cause pathological changes including oedema of the heart, for example (Kudayer, Alsandaqchi, Saleh, & Alwan, 2019).

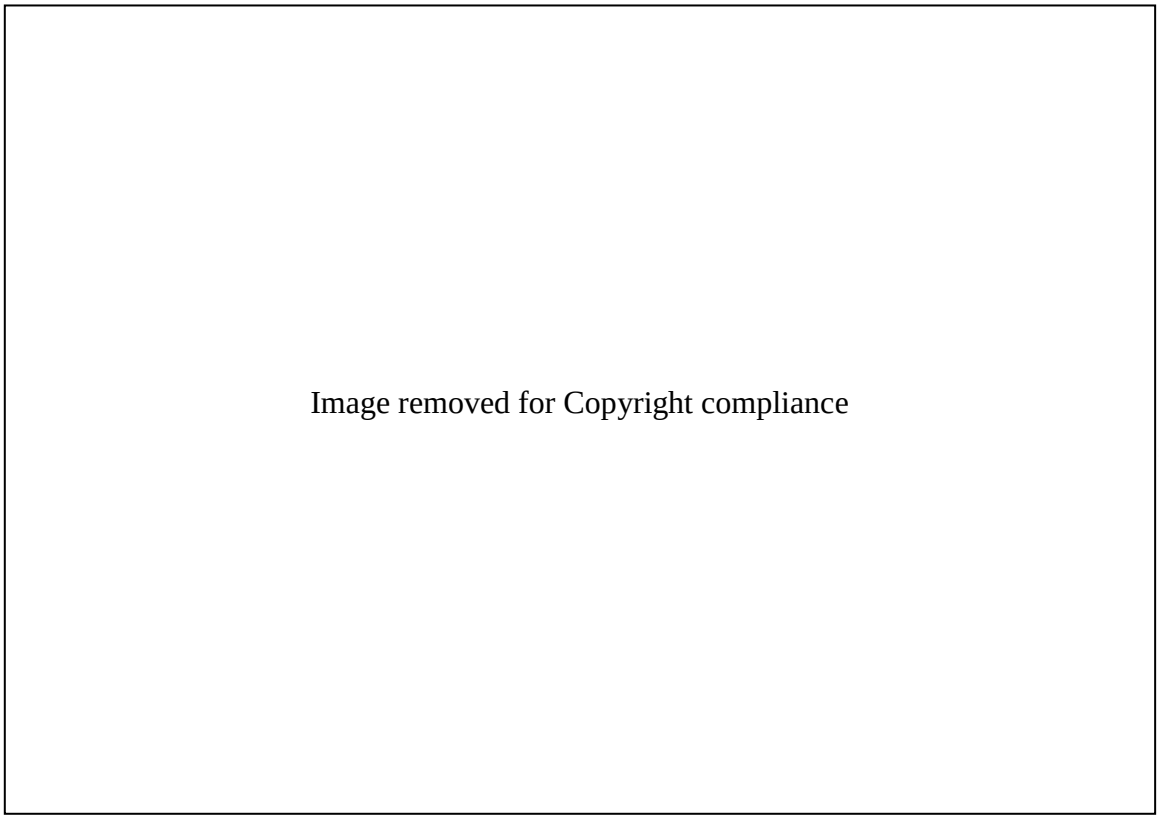

Figure 1. Targets for intervention in populations at high risk for liver cancer.
Source: Groopman, Kensler, and Wild (2008)

2.3.4. Immunomodulation

Several studies have shown that aflatoxin exposure affects the human immune system. In a study in Ghana, among many cells of the immune system response, only a few were active in subjects exposed to high dietary aflatoxin levels (Jiang et al., 2005). Similarly, lower concentrations of secretory IgA in the saliva of Gambian children were associated with high concentrations of serum aflatoxin-albumin adducts (Turner, Moore, Hall, Prentice, & Wild, 2003). Jiang et al. (2008) reported that both in HIV-positive and HIV-negative individuals in Ghana, lower concentrations of blood CD4+ T regulatory cells, naïve CD4+ T cells and lower B-cell counts were associated with high concentrations of plasma aflatoxin-albumin adducts.

2.4. Control methods and prevention of aflatoxins

To reduce the mycotoxin concentration in food and feed, different methods have been proposed. These methods should meet criteria like (i) possibility of destroying fungal spores, (ii) technological properties of the product should not significantly alter the nutritive value of the food or feed and retain the nutritive value to an acceptable value, and (iii) no chemical residues should be left in food or feed during removal, destruction or inactivation of the mycotoxins (Temba et al., 2016).

For food safety, it is necessary to identify, analyse and completely remove any aflatoxins from food. Different methods can be used to identify and detect aflatoxins, which include the official analytical procedures suggested by the International Association of Official Analytical Chemists. As aflatoxins are not found on the surface of the product, it is difficult to remove aflatoxins from a commodity. Aflatoxins can be removed by binding with clay (Phillips et al., 2008). Alkaline oxidizing agents like sodium hypochlorite have shown excellent reactivity with aflatoxin. These reagents are applied to the surfaces of laboratory glassware or benches for aflatoxin inactivation, but it is difficult to use this procedure to solve the food contamination problem. There are factors that increase the production of aflatoxin, like improper storage condition of crops, inadequate drying, the existence of dry weather near crop maturity and high moisture content during harvest. In addition, postharvest conditions, for example, processing, storage, and transport, also increase the risk of crop contamination (Kumar, 2018).

Different methods and approaches have been used to control aflatoxins and, among these, prevention of crop fungal growth is the most important. Therefore, during processing, storage, and harvesting of crops and feedstuffs, hygienic safety measures should be followed. Effective drying tools and proper storage operations of crops can minimise fungal growth (Rachaputi, Krosch, & Wright, 2002). In comparison to dry fruits and nuts that have been stored in shells, those stored without shells show higher aflatoxin concentrations (Iqbal et al., 2018). In Turkey, providing technical assistance, strict quality control measures and implementation of regulatory initiatives have reduced aflatoxin contamination (Basaran & Ozcan, 2009).

Physical, biological and chemical methods have been used to prevent aflatoxin contamination of food/feed. Washing, crushing with dehulling and sorting are examples of physical methods (Temba et al., 2016). Radiation, roasting, cooking, and heat or separation of the contaminated fractions are physical means of either inactivation or removal of aflatoxins (Khoshpey, Farhud, & Zaini, 2011). The aflatoxin concentration in foods such as maize can be reduced by heat treatment procedures like steaming, boiling, roasting and baking (Jalili & Scotter, 2015). The resistance of AFM_1 to thermal inactivation causes difficulty in controlling of AFM_1 in milk by using simple autoclaving and pasteurization; therefore, other options like ultra-high temperature processing (UHT) is recommended (Movassagh, 2011).

2.4.1. Decontamination of aflatoxins

There was a reduction in aflatoxins and the mould flora when dried figs are irradiated with UV-rays. Coconut agar medium was used to test the aflatoxin-producing ability of aflatoxigenic strains and *A. flavus* and *A. parasiticus* agar media were used to detect aflatoxigenic species. The reproduction ability of the moulds in dried figs was depressed by UV-radiation. Additionally, an increase in exposure to UV-radiation decreased mould count (Isman & Biyik, 2009).

Adsorption can be used to reduce the aflatoxin concentration. During digestion, the toxins become bound to adsorbents in the gastrointestinal tract. Complex carbohydrates, synthetic polymers, active carbon and diatomaceous earth are adsorbents used for this purpose. Zeolite, activated charcoal and yeast either alone or in combination with adsorbents in broiler diets

can reduce aflatoxin toxicity (Huwig, Freimund, Käppeli, & Dutler, 2001; Khadem, Sharifi, Barati, & Borji, 2012).

To reduce aflatoxin contamination, chemical methods are used such as fungicides like prochloraz, propiconazole, epoxyconazole, tebuconazole, cyproconazole, oltipraz, chlorophyllin and azoxystrobin (Haidukowski et al., 2005; Ni & Streett, 2005). Pistachio is a nut with a good taste and high demand in the world. However, it is easily contaminated by *A. flavus* during post-harvest processing, even at low moisture. As chemical disinfectants can result in negative effects on human health and the environment, herbal plant extracts like cloves and cinnamon have been used on pistachio kernels to inhibit fungal growth. Nuts can be stored for more than a month without unwanted side-effects by using herbal extracts (Khorasani, Azizi, Barzegar, Hamidi- Esfahani, & Kalbasi- Ashtari, 2017). *Ocimum tenuiflorum* (holy basil) can significantly inhibit aflatoxin production on rice. It also used for the degradation of different toxins in feed and food (Panda & Mehta, 2013). Addition of 4% garlic extract in aflatoxin-contaminated chicken feed improved egg production and weight gain in 25 laying hens. Garlic extracts reduced aflatoxin residues by 42.2% in laying hens treated with 0.4 mg/kg of AFB_1 and by 49% in the group treated with 5mg/kg of AFB_1 (Maryam, Sani, Juariah, & Firmansyah, 2003).

A randomised control trial was conducted on 300 Tanzanian children using intervention methods to reduce the contamination of aflatoxins. These included (i) hand sorting, (ii) appropriate sun drying, (iii) drying maize on the mat/raised platforms, (iv) de-hulling before milling and (v) application of insecticides during storage. The maize samples were collected from the children's houses during the trial and at 6 months of harvesting. Bodyweight measurements and 24 h dietary recall was performed after 6 months of harvesting. The results showed higher mean aflatoxin concentrations in the control than in the intervention group. At the end of the intervention, the estimated mean intake of aflatoxin was higher in control than in the intervention group. There was also a 67% lower prevalence of underweight children in the intervention group than in the control group. The difference between the groups' mean weight-for-age Z-score was 0.57 (95% CI; 0.16,-0.98; $p = 0.007$) (Kamala et al., 2018).

Hydrolytic agents (alkalis and acids), chlorinating agents (gaseous chlorine, chlorine dioxide, and sodium hypochlorite) and oxidising agents ($NaHSO_4$, O_3, and H_2O_2) can also degrade aflatoxins. The hydrolytic agents cause the oxidation of the lactone ring or the double bond of the terminal furan ring of aflatoxin. Benzoyl peroxide, aldehydes, 75% methanol, 5%

dimethylamine hydrochloride, aldehydes, osmium tetroxide, iodine, ferrous ammonium sulphate, potassium permanganate, quinones, sodium borate and formaldehyde are chemicals that have been used for degradation of aflatoxins. Because of their residues, there is limited use of these chemical methods (Kumar, 2018).

Washing of seeds with water can remove up to 40% AFB_1. It must be ensured that the nutritional value is maintained during the degradation process when detoxification procedures like physical, chemical and microbiological methods are employed. Additionally, they should not create toxic degradation substances or chemicals that can cause mutagenesis/ carcinogenesis and should ensure the destruction of *Aspergillus* spores and mycelia (Kumar, 2018). Organic acids like citric acid can also be used to detoxify aflatoxins. For example, 1 M citric acid can convert up to 97% of AFB_1 into AFB_2a, which has lower toxicity. Boiling AFB_1 with citric acid for 20 minutes increases this transformation to 98% (Rushing & Selim, 2016).

Biological methods can also be applied, such as the use of an atoxigenic fungi that can compete with toxigenic fungi and decrease their environmental toxicity. A reduction of aflatoxin contamination from 74.3% to 99.9% in peanuts from the United States was observed following the use of atoxigenic strains of *A. flavus* and *A. parasticus* in the soil of developing crops (Dorner, Cole, & Blankenship, 1998). Fungi and yeast can also be used to degrade aflatoxins in solutions. Complete removal of aflatoxin from contaminated corn, milk, peanut butter, and peanuts has been achieved by using bacteria (Kumar, 2018).

Probiotic and yeast mixtures can also be used to eliminate aflatoxins from food. These probiotics and yeast mixtures can be applied as food additives to decrease the bioavailability of aflatoxins (Hamad, Zahran, & Hafez, 2017). It was reported that a probiotic like *Lactobacillus casei Shirota* (Lcs) has the potential to bind to aflatoxin and therefore, can be used as a preventive agent. Lcs reduced the stunting in animals exposed to AFB_1. Additionally, there was a reduction in serum AFB_1 in animals treated with Lcs compared with the controls, without Lcs (Winnie-Pui-Pui Liew, Adilah, & Sabran, 2018).

Probiotic yoghurt with *Streptococcus thermophilus, Lactobacillus rhamnosus* GR-1 and *Weissella cibaria* NN20 reduced AFB_1 concentration in the urine of Kenyan children. A significant reduction in the urinary concentration of AFB_1 was observed in children after 7 days of treatment with probiotic yoghurt but not in the control group (Nduti et al., 2016). Three probiotics, namely, *Saccharomyces cerevisiae* var. *boulardii*, *S. cerevisiae* UFMG 905

and *Lactobacillus delbrueckii* UFV H2b20, were used to evaluate their effects on aflatoxin production in peanut grain. The aflatoxin production was reduced by 72.8% for *S. boulardii* and 65.8% for *S. cerevisiae.* When inoculated with probiotic combinations, 96.1% reduction was observed with *S. boulardii* plus *L. delbrueckii*, 71.1% with *S. boulardii* plus *S. cerevisiae* and 66.7% with *L. delbrueckii* plus *S. cerevisiae* (Silva et al., 2015).

2.5. Aflatoxins detection methods

2.5.1. Metabolism and biomarkers of aflatoxins

The metabolic reactions for AFB_1 produce a range of metabolites, namely, hydroxylation to AFM_1 and AFQ_1, reduction to aflatoxicol (AFL), demethylation to AFP_1 and epoxidation to AFB_1-8,9-epoxide (Leong, Latiff, Ahmad, & Rosma, 2012). Epoxide is the most reactive metabolite, believed to be responsible for both acute and chronic toxicity. AFB_1 can exist in the form of endo and exo-epoxide (Figure 2). The latter is highly electrophilic and forms covalent bonds at the N-7 guanine residue leading to depurination and it reacts with DNA guanine moiety to cause carcinogenesis. Depurination is “a process where the purine base of DNA molecule is lost, potentially leading to a somatic mutation and carcinogenesis” (Fung & Clark, 2004). CYP3A4, 3A5, 3A7 and 1A2 are the major cytochrome P450 (CYP) enzymes involved in the metabolism of aflatoxin and the liver is the predominant site of metabolism. Aflatoxin can cross the placenta, and CYP3A7, the major CYP in the human foetal liver, can activate AFB_1 to 8,9-epoxide (Wild & Gong, 2009).

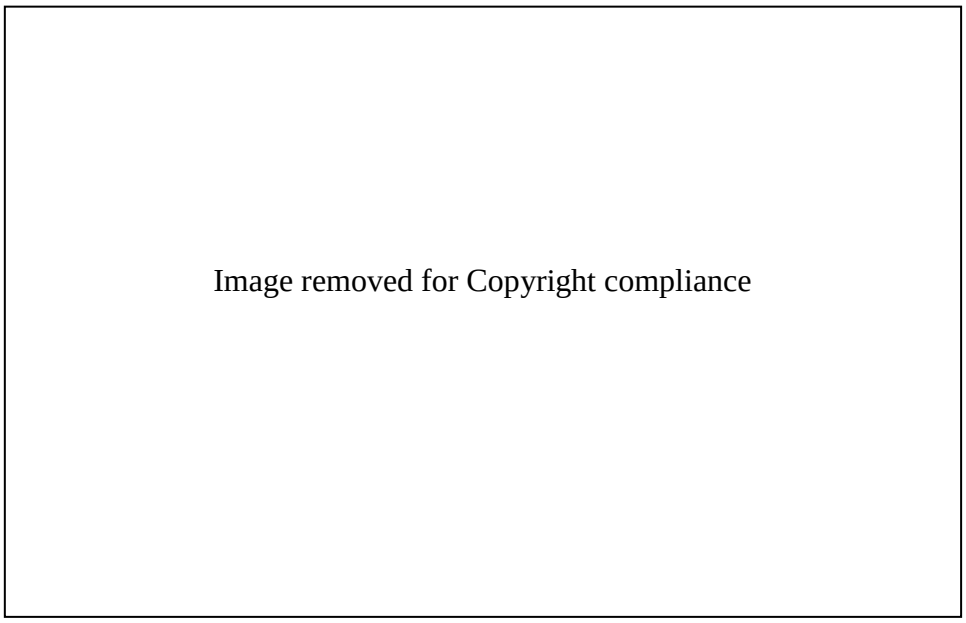

Figure 2. Aflatoxin B_1 metabolism.
Me, methyl; GST, glutathione S-transferase; SG, glutathione; MA, mercapturic acid.
Source: Turner et al. (2012)

Aflatoxins and its metabolites can be detected in human body fluids. In humans, urinary aflatoxins (B_1, B_2, G_1, G_2, Q_1, P_1, and B_2a), aflatoxin-N7-guanine and aflatoxicol have been detected. Aflatoxin-albumin adduct and AFB_1-lys adduct are the main metabolites of aflatoxin that are detected in human blood. AFM_1 has been detected in human breast milk. Additionally, aflatoxins have been detected in faeces, nasal secretions, sputum and tissue biopsies (lung, brain, liver) of individuals exposed to aflatoxins. ELISA, RIA, IAC, HPLC and LC-MS/MS are the major analytic techniques currently available to measure different metabolites of aflatoxins (Leong, Latiff, et al., 2012).

2.5.2. Techniques of aflatoxin analysis

Analytical methods for mycotoxin detection (Figure 3) start with toxin extraction from the matrix by using an adequate extraction solvent. A clean-up step is usually proposed to prevent interference from the extract. The final step is the detection of toxins by using appropriate analytical methods (Leslie, Bandyopadhyay, & Visconti, 2008).

Commonly used chromatographic methods for the quantitative determination of mycotoxins are fluorescence or mass spectrometry (MS), gas chromatography coupled with electron capture, high-performance liquid chromatography (HPLC) coupled with ultraviolet (UV). Additionally, membrane-based immunoassays or enzyme-linked immunosorbent assays (ELISA) are commercial immunometric assays that have been used for aflatoxin screening (Leslie et al., 2008).

Mycotoxins analysis has been greatly advanced by coupling liquid chromatography techniques with mass spectrometry (LC-MS/MS) (Abdallah, Girgin, Baydar, Krska, & Sulyok, 2017; Monbaliu et al., 2009), which is a highly sensitive and reliable method (Zhao et al., 2015). In addition, it also helps to analyse multiple mycotoxins (Abdallah et al., 2017). High-performance liquid chromatography-fluorescence (HPLC-FLD) coupled with an efficient extraction and clean-up method is the frequently used non-mass spectrometry chromatographic technique. It has been used for quantitative analysis of mycotoxins, mainly aflatoxins. The sensitivity of HPLC-FLD is similar to that of the LC-MS/MS method, but it is appropriate for the analysis of single mycotoxins (Alshannaq & Yu, 2017).

Table 2.Advantage and disadvantage of different mycotoxin analysis methods

Method	**Advantage**	**Disadvantage**
TLC	Simple, inexpensive and rapid	Poor sensitivity (for some mycotoxins)
	Can be used for screening	Poor precision
	Simultaneous analysis of multiple mycotoxins	Adequate separation may require two-dimensional analysis
	Sensitive for aflatoxin and ochratoxin A	Quantitative only when used with a densitometer
GC	Simultaneous analysis of multiple mycotoxins	Expensive equipment
	Good sensitivity	Specialist expertise required
	May be automated (autosampler)	Derivatization required
	Provides confirmation (MS detector)	Matrix interference problems
		Non-linear calibration curve
		Drifting response
		Carry-over effects from previous sample
		Variation in reproducibility and repeatability

	Good sensitivity	Expensive equipment
	Good selectivity	Specialist expertise required
	Good repeatability	May require derivatization
HPLC	May be automated (autosampler)	
	Short analysis times	
	Official methods available	
LC/MS	Simultaneous analysis of multiple mycotoxin	Very expensive
	Good sensitivity (LC/MS/MS	Specialist expertise requested
	Provides confirmation	Sensitivity relies on ionization technique
	No derivatization required	Matrix assisted calibration curve (for quantitative analysis)
ELISA	Simple sample preparation	Cross-reactivity with related mycotoxins
	Inexpensive equipment	Matrix interference problems
	High sensitivity	Possible false positive /negative results
	Simultaneous analysis of multiple samples	Confirmatory LC analysis required
	Suitable for screening	Critical quantitation near regulatory limits
	Limited use of organic solvents	Semi-quantitative (visual assessment)
	Visual assessment	
Rapid tests	Simple and fast (5-10min)	Qualitative or semi-quantitative (cut off level)
	No expensive equipment required	Possible false positive/negative results
	Limited use of organic solvents	Cross-reactivity with related mycotoxins
	Suitable for screening purposes	Matrix interference problems
	Can be used *in situ*	Lack of sensitivity near regulatory limits

TLC= Thin Layer Chromatography; GC= Gas Chromatography; HPLC= High Performance Liquid Chromatography; LC/MS= Liquid Chromatography/Mass Spectrometry; ELISA= Enzyme-Linked Immunosorbent Assay; Rapid tests= membrane based card test; antibody-coated tube; immune dot cup test

Source: Leslie et al. (2008)

The enzyme-linked immunosorbent assay (ELISA) is probably the most commonly used method for mycotoxin determination among all published immunological based techniques (Alshannaq & Yu, 2017). Currently, ELISA methods that use monoclonal or polyclonal

antibodies against mycotoxins are commercially available for qualitative, semi-quantitative and quantitative analysis of the main mycotoxins in food matrices (Leslie et al., 2008). It is specific, rapid and relatively easy method (Shiu, Wang, & Yu, 2010). The disadvantage of ELISA is the possibility of cross-reactivity and its dependence on a specific matrix. The detection of a single mycotoxin is performed by a one-specific ELISA kit. Therefore, to test a sample for multiple mycotoxins requires more kits and can become quite expensive. High-performance liquid-chromatography tandem mass spectrometry (HPLC-MS/MS) is the gold standard quantitation method for accurate detection of mycotoxins (Kleigrewe, Niehaus, Wiemann, Tudzynski, & Humpf, 2012).

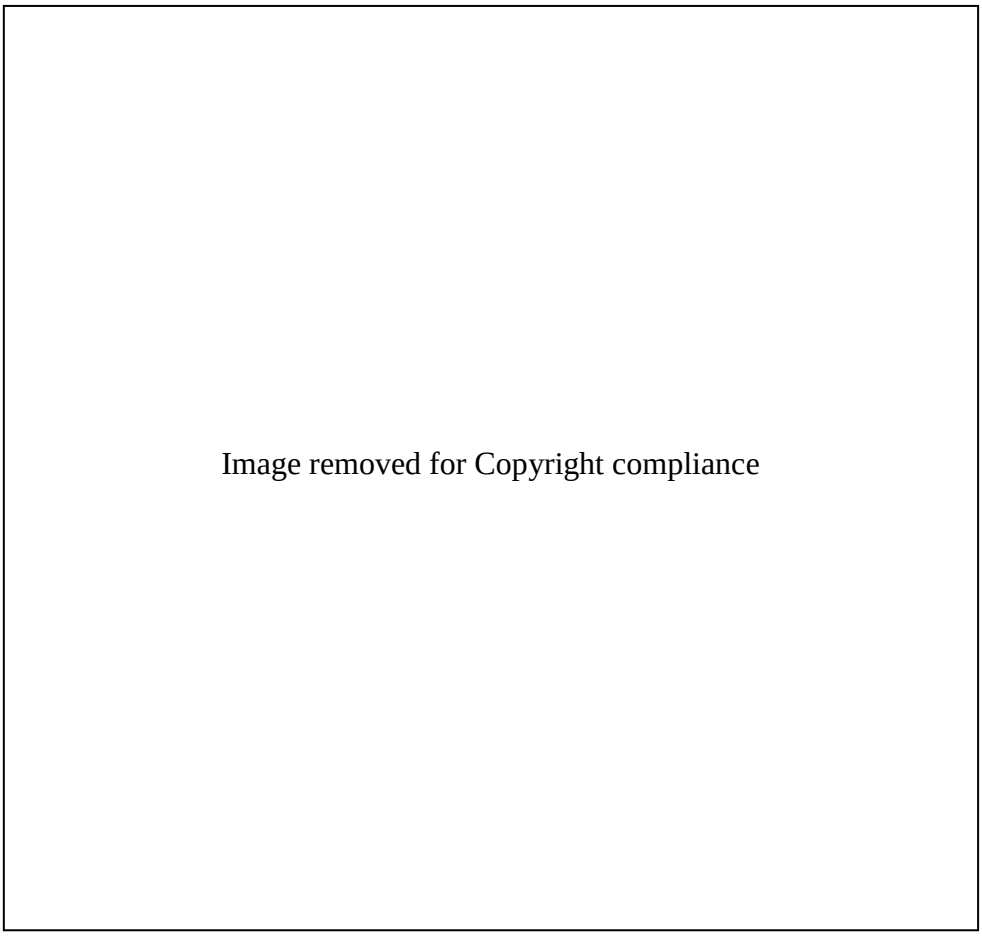

Figure 3. Flow diagram of steps commonly used in analysis of food commodities for mycotoxins.
Source: Alshannaq and Yu (2017)

Chapter 3
Materials and Methods

3.1. Study area

Ethiopia is administratively divided into two independent city administrations and nine regions. The current study was conducted in five rural districts (Manna, Seka Chekorsa, Shebe Senbo, Kersa, and Omo-Nada) of the Oromia Region. Oromia Region is the largest province with 353, 0006 km^2 of land area (32% of the country) with a population of 27,158,471 (36.7% of the country). The temperature of the region ranges from 7 to 30°C, with an annual average rainfall of 1600 mm and elevation of 800–3360 m above sea level. About 90% of the people in this region were rural residents and agriculture is the primary source of income (CSA, 2008).

3.2. Data collection and sampling

Data collection and blood sampling were conducted from November–December 2018 from the five districts of the Oromia Region of South-western Ethiopia (Figure 4). The recruitment of the study population was made with support from the health extension workers. The information about the research was distributed to the population through the health extension workers in each district. The selection of the study population was made on a volunteer basis with the random selection of one individual per household. A total of 100 healthy individuals participated in the study, 20 per district, with an equal number of males and females within the age range 18–60 years. A research information sheet was distributed to all participants. For non-educated groups, the research information sheet was explained by the health extension workers. Once people decided to participate in the study, they were requested to sign a consent form before the beginning of the sample and data collection. The research comprised two parts, a survey and a laboratory analysis of the samples. A questionnaire was prepared to obtain information on socio-demographic characteristics (residential area, age, gender, occupation, educational level, marital status) and weekly-based food frequency data of the participants (Appendix A).

After the survey, participants were requested to visit a nearby health centre for blood collection and weight and height measurement. The weight and height were used to calculate

the BMI. Then a 6-ml blood sample was collected from each participant by a laboratory technician at the health centres. Blood was allowed to clot, and the serum separated following centrifugation. Approximately 1 ml of serum was transferred into three Eppendorf tubes. On the same day, the Eppendorf tubes containing serum were transported to the Jimma Referral Hospital laboratory using an icebox and stored at −80°C. Two Eppendorf tubes in dry ice were sent for aflatoxin analysis to the Centre of Excellence in Mycotoxicology and Public Health, Faculty of Pharmaceutical Sciences, Ghent University, Belgium (Appendix B). The other sample was stored at Jimma Referral Hospital laboratory at −80°C in case the analysis was to be repeated.

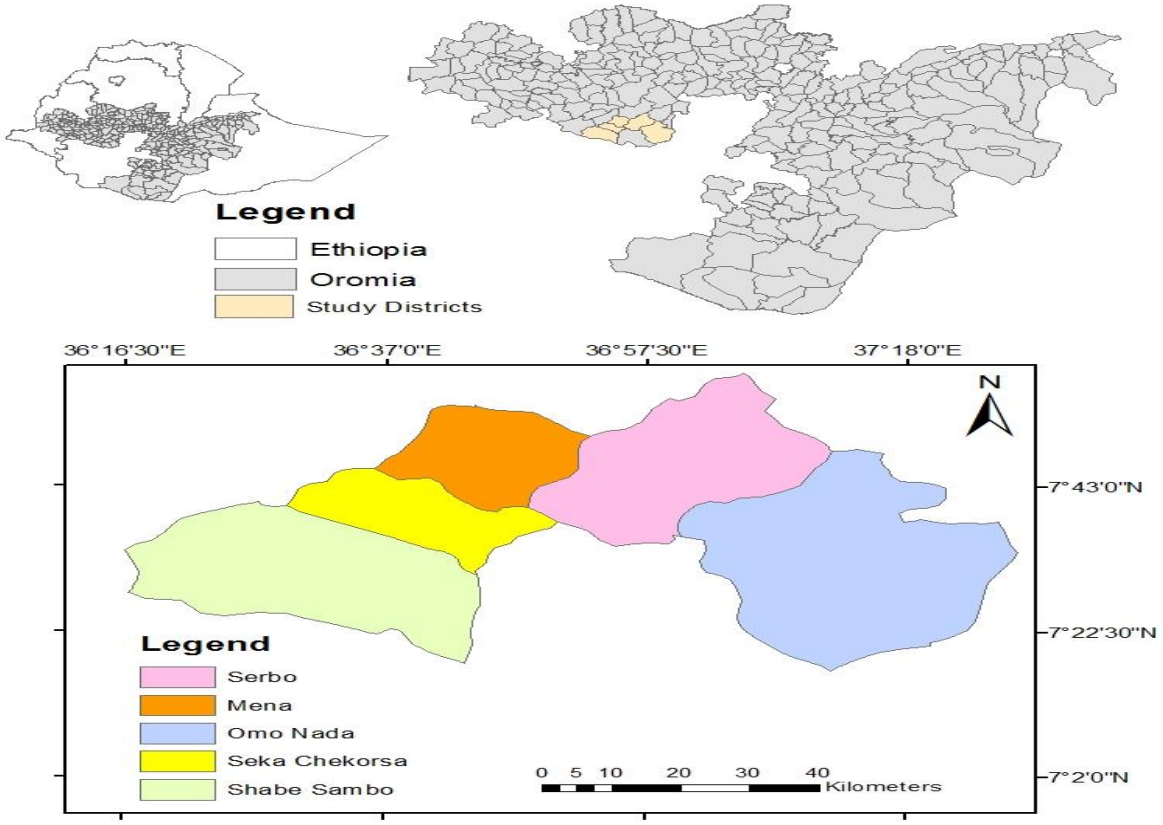

Figure 4. Location of the study area.

3.3. Laboratory analysis

Serum aflatoxins and its metabolites (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFB_1-lys), as biomarkers of exposure to aflatoxins were analysed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Isotope-labelled $^{13}C_{15}$ aflatoxin was used as an internal standard.

3.3.1. Materials and reagents

The individual mycotoxin standards (1000 µg/mL), aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1) and $^{13}C_{17}$-aflatoxin B_1 ($^{13}C_{17}$-AFB_1) (internal standard) were purchased from Sigma Aldrich (Bornem, Belgium). AFB_1-lys standards were kindly supplied by Carleton University, Ottawa, Canada. The working solutions of AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFB_1-lys and $^{13}C_{17}$-AFB_1 (10 µg/mL) were prepared in methanol, stored at −18°C, and renewed monthly. Water used was Milli-Q® SP Reagent water system (Millipore Corp., Brussels, Belgium). Methanol (LC-MS grade) was purchased from BioSolve (Valkenswaard, The Netherlands), acetonitrile from Analar Normapur and ammonium acetate from VWR International (Zaventem, Belgium). Acetic acid (glacial, 100%) was from Merck (Darmstadt, Germany). Formic acid analytical grade (98–100%) and sodium chloride (> 99.5%) were from Merck (Darmstadt, Germany). Ultrafree®-MC centrifugal filter devices (0.22 µm) were purchased from Millipore (Bedford, MA, USA).

3.3.2. Sample preparation for measurement of aflatoxins

Serum samples were initially thawed and acetonitrile (A_CN) used for protein precipitation. One hundred microlitres of A_CN and 100 µL of serum samples were transferred into Eppendorf tubes. The mixture was centrifuged (4,000 g, 15 min) to create two layers, a large aqueous layer on top and a circular flake of proteins at the bottom. An aliquot (160 µL) of the supernatant was carefully transferred to glass tubes and evaporated to dryness using a TurboVap 40°C. The samples were reconstituted with 80 µL of injection solvent and transferred to the centrifugal filter tubes. The samples were transferred from tubes to the injection vials and spiked with 5 µL of an internal standard prior to analysis. Finally, the serum samples were analaysed in pentaplicate. For each batch, six standards and one blank

calibrator were prepared for the calibration curve. The blank contained 80 μL of internal standard ($^{13}C_{17}$-AFB_1) and 120 μL injection solvent.

3.3.3. LC-MS/MS analysis

LC-MS/MS analyses were performed using an Acquity UPLC coupled to a Xevo TQ-S (Waters, Manchester, UK), equipped with a positive electrospray ionisation source (ESI). Two mobile phases were used. Mobile phase A was 95% water and 5% methanol. Mobile phase B 95% was methanol and 5% water. There was also an addition of 5 mM of ammonium acetate and 0.1% formic acid in both phases. The gradient elution program started with 100% mobile phase A. After an isocratic phase for 0.5 min at initial conditions, mobile phase B was increased to 37% in 2.5 min. Then, during the next 13 min, phase B reached 75%. Later, for 2 min, it was enhanced with 100% mobile phase B. An equilibration step of 1.5 min was introduced resulting in a total run time of 19.5 min. The flow rate was set at 0.4 mL/min. The mass spectrophotometer was operated in positive electrospray ionisation mode (ESI^+). The capillary voltage was 30 kV, and nitrogen was applied as the spray gas. The source and de-solvation temperatures were set at 150°C and 200°C, respectively. The argon collision gas pressure was 9×10^{-6} bar, cone gas flow 50 L/h and the de-solvation gas flow was 500 L/h. Two selected reaction monitoring transitions with a specific dwell-time were optimised for each analyte, in order to increase the sensitivity and the selectivity of the mass spectrometric conditions.

3.3.4. Method validation

The LC-MS/MS method developed was successfully validated based on the European Commission Decision 2002/657/EC rules for the analytical methods to be used in the testing of official samples. Matrix-matched calibration plots were constructed for the determination of the analytes. MassLynx 4.1 and TargetLynx 4.1 software (Micromass, Manchester, UK) were used for data acquisition and processing. The real compounds were identified using peak ratio (relative ion intensity), the retention time and the signal to noise ratio (Commission of the European Communities, 2002). After the completion of all criteria, the response is expressed as the ratio of the compound divided by the ratio of the area of the internal standard.

Specificity was checked to ensure that there was no interference or any peaks for the identification and quantification of the target compounds in the ±2.5% margin of the relative time in five blank samples. Evaluating the linearity, the homogeneity of variance was checked before fitting the linear model. The linearity was interpreted graphically using a scatter plot with the $r^2 \geq 0.95$. Recovery was calculated after measuring the concentration and the actual (spiked) concentration. The observed concentration was calculated in triplicate from a matrix-matched calibration curve. The precision was calculated in terms of the intra-day (RSD_r) and inter-day precision (RSD_R). Limit of detection (LOD) was calculated as three times the standard error of the intercept, divided by the slope of the standard curve; the limit of quantification (LOQ) was similar, differing by six times the standard error. The calculated LOD and LOQ, which should be more than 3 and 10 respectively, were verified by the signal-to-noise ratio (S/N), according to the IUPAC guidelines. The results of the performance characteristics of the LC-MS/MS method agreed with the criteria outlined in the European Commission Decision 2002/657/EC (Table 1). Briefly, four identification points should be fulfilled to allow confirmation of the identity of the detected compound, one precursor and at least two product ions should be monitored, the relative intensities of the detected ions should correspond within accepted deviations to those of the calibration, detected ions should have a signal-to-noise ratio (S/N) of at least 3 and the relative retention time of the detected ions must range within a margin of 2.5%.

Table 3.Validation of aflatoxin metabolites (used as biomarkers of aflatoxin exposure)

Method validation Parameters	**AFB_1**	**AFB_2**	**AFG_1**	**AFG_2**	**AFM_1**	**AFB_1-lys**
Range (µg/L)	0.015-2.00	0.015-2.00	0.015-2.00	0.015-2.00	0.015-2.00	
Cut-off (x)	0.5	0.5	0.5	0.5	0.5	
LOD (µg/L)	0.017	0.005	0.005	0.0002	0.004	0.015
LOQ (µg/L)	0.064	0.022	0.018	0.007	0.011	0.035
MU (0.25x) (%)	121.2	119.9	114.3	109.5	115.9	
MU (0.5x) (%)	117.4	115.1	111.5	109.9	118.7	
MU (1x) (%)	113.9	114.1	112.3	114.8	114.4	
MU (2x) (%)	104.8	106.7	107.1	103.9	106.9	
MU (4x) (%)	106.7	107.9	103.1	99.7	107.2	

LOD = Limit of detection; LOQ = Limit of quantification; MU= Maximum limit

3.4. Statistics

Statistical analysis of data was performed using the software SPSS version 20. The serum aflatoxin concentrations among districts, socio-demographic groups and BMI groups were compared using an ANOVA test. Pearson correlation was used to assess the correlation between serum aflatoxin concentration with food frequency and BMI. p-values of <0.05 were considered as statistically significant.

3.5. Ethical clearance

Ethical approval (Appendices C and D) for this study was granted by Lincoln University and Jimma University, Ethiopia. The objective of the research was explained to the participants and written consent (Appendices E–G) was obtained from each participant before the collection of data and blood samples.

Chapter Four

Results

4.1 Aflatoxin concentrations in human serum

One hundred serum samples (Appendix H) from 100 adults with the age range of 18–60 were analysed for six aflatoxins; AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFB_1-lys. One or more aflatoxins were detected in 64/100 (64%) of serum samples collected from five districts (Shebe Senbo, Manna, Seka Chekorsa, Omo Nada and Kersa) of South-western Ethiopia. In the 64 aflatoxin-positive samples, 2–5 aflatoxins co-occurred in 44 (69%) of samples. The total aflatoxin concentration at the individual level was within the range of 9.4 – 465.6 ng/L for males and 11.4 – 462.4 ng/L for females. The most frequently detected aflatoxin was AFM_1 (38%) > AFB_1 (37%) > AFB_1-lys (27%) > AFG_1 (25%) > AFG_2 (25%) > AFB_2 (5%). The mean aflatoxin concentrations were 72.3, 69.9, 58.6, 43.2, 36.9 and 6.1 ng/L for AFB_1-lys, AFM_1, AFG_2, AFB_1, AFG_1, and AFB_2, respectively. The serum aflatoxin occurrences and concentrations are given in Table 4**.**

Table 4.Incidence and concentrations of aflatoxins in serum samples of adults collected from South-western Ethiopia

Aflatoxins	**Number of positive samples**	**Serum aflatoxin concentration: mean (range) (ng/L)**
AFB_1	37 (37%)	43.2 (<LOD–422.9)
AFB_2	5 (5%)	6.1 (<LOD–8.3)
AFG_1	25 (25%)	36.9 (<LOD–126.8)
AFG_2	25 (25%)	58.6 (<LOD–152.5)
AFM_1	38 (38%)	69.9 (<LOD–210.1)
AFB_1-lys	27 (27%)	72.3 (<LOD–130.0)

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 and AFB_1-lys: Aflatoxin B_1-lysine. LOD: Limit of detection. The means are the average of positive samples.

4.2. Correlation of serum aflatoxin concentrations with socio-demographic characteristics, body mass index, and food frequency data

A total of 100 serum samples were analysed from five districts, with 20 samples (10 females and ten males) from each district. The frequency of occurrence of aflatoxin was highest in Shebe Senbo district (17/20; 85%), followed by Manna (15/20; 75%), Seka Chekorsa (12/20; 60%), Kersa (11/20; 55%) and Omo Nada (9/20; 45%). The highest mean total aflatoxin concentration was detected in Omo Nada district (175.2 ng/L) > Manna (156.0 ng/L) > Shebe Senbo (151.0 ng/L) > Seka Chekorsa (98.3 ng/L) > Kersa (63.8 ng/L). The mean total serum aflatoxin concentrations among districts were not significantly different ($p = 0.2107$), but the mean AFB_1-lys concentration was significantly different ($p = 0.0009$). Figures 5 and 6 present the total mean aflatoxin concentrations and the mean AFB_1-lys concentrations among districts, respectively. Table 5 shows the concentrations of aflatoxins in each district.

Table 5.Aflatoxin occurrence and mean concentrations in serum of adults from five districts of south-western Ethiopia

District	**Number of samples analysed**	**Number of positive samples (%)**	**Mean serum aflatoxin concentration in ng/L (number of samples)**					
			AFB_1	AFB_2	AFG_1	AFG_2	AFM_1	AFB_1-lys
Seka Chekorsa	20	12 (60%)	25.1 (8)	<LOD	14.9 (4)	53.0 (2)	31.0 (8)	94.3 (6)
Omo Nada	20	9 (45%)	23.0 (4)	8.3 (1)	41.0 (5)	40.2 (7)	103.2 (6)	61.8 (6)
Kersa	20	11 (55%)	49.4 (10)	<LOD	1.4 (1)	34.2 (1)	39.7 (2)	93.1 (1)
Manna	20	15 (75%)	15.6 (2)	7.0 (1)	59.0 (10)	121.1 (8)	108.4 (10)	57.4 (10)
Shebe Senbo	20	17 (85%)	58.6 (13)	5.0 (3)	40.5 (5)	70.5 (7)	84.7 (12)	102.1 (4)

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 and AFB_1-lys: Aflatoxin B_1-lysine. LOD: Limit of detection. The means are the average of positive samples.

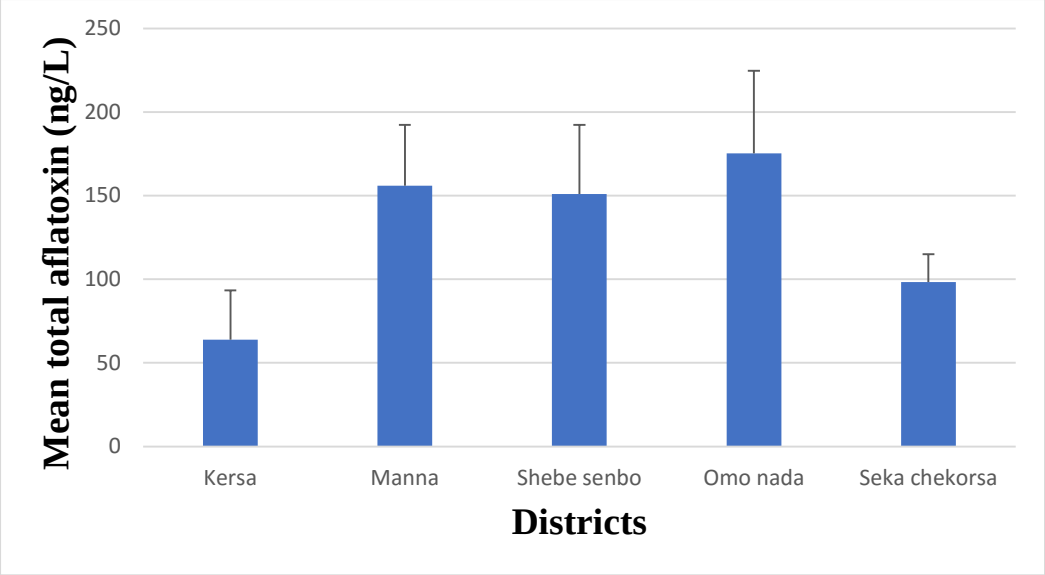

Figure 5. Total aflatoxin concentrations (mean ± SE; p = 0.2107) in the people sampled (n = 20) from each of the five districts in the Oromia Region, Ethiopia.

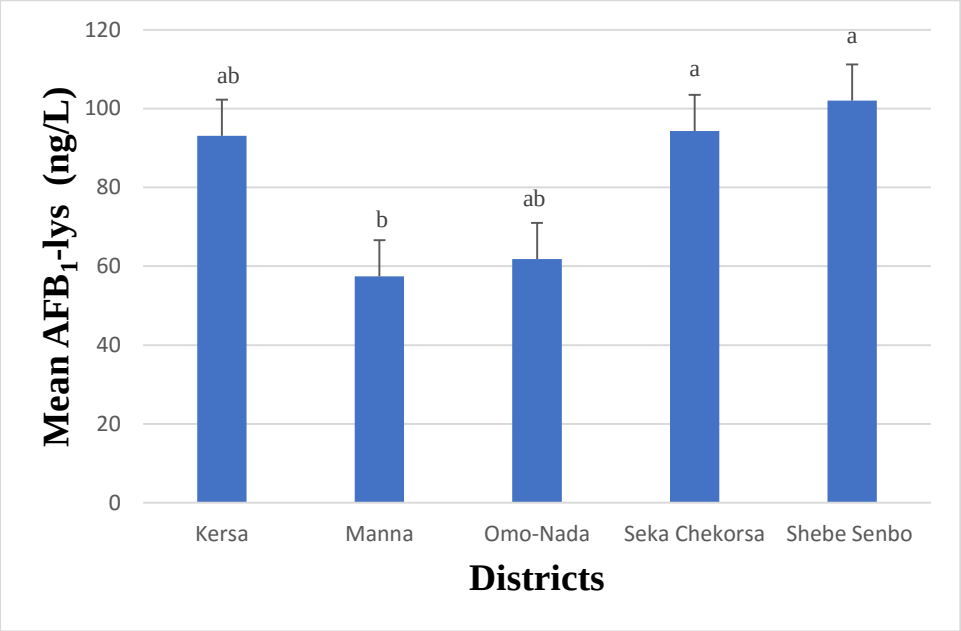

Figure 6. AFB_1-lys concentration (mean ± SE; p = 0.0009) in the sampled population in the five districts in the Oromia Region, Ethiopia.

The mean concentrations of total serum aflatoxins were analysed in relation to the socio-demographic group, BMI and frequency of food consumption (Appendix I). The highest mean concentration of total serum aflatoxin was detected in the 31–40 age group (140.6 ng/L) > 51–60 group (139.5 ng/L) > 18–30 group (132.6 ng/L) > 41–50 (93 ng/L). The mean total serum aflatoxin concentration in males was higher than in females, 132.5 and 126.5

ng/L, respectively. In terms of education, those with primary education had the highest concentration of 210 ng/L > informal education (133.1 ng/L) > secondary education (108.9 ng/L) and was least among those with tertiary education (69.2 ng/L). The contamination in married people (130.5 ng/L) > singles (126.8 ng/L). The mean total serum aflatoxin concentration in students (144.0 ng/L) was higher than in other occupations, followed by farmers (141.8 ng/L) > housewives (139.3 ng/L) > others (86.5) > office workers (64.1 ng /L). Figures 7–11 show the mean total aflatoxin concentration for these different socio-demographic groups. The mean total aflatoxin concentration was not associated with any of the socio-demographic characteristics: age (p = 0.8354), gender (p = 0.6609), education (p = 0.2295), marital status (p = 0.7061), or occupation (p = 0.5867).

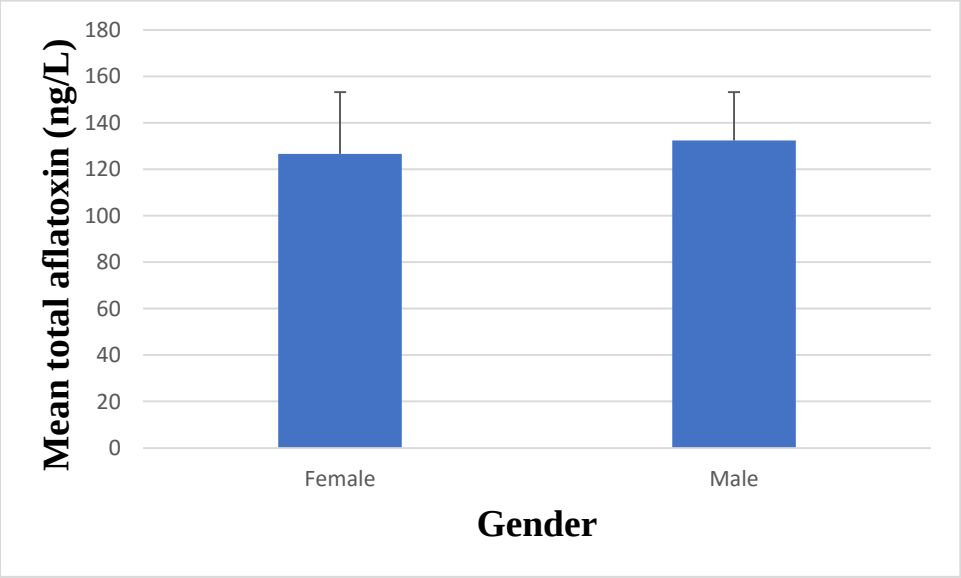

Figure 7. Total aflatoxin concentration (mean ± SE; p = 0.6609) in the sampled population based on gender of the participants.

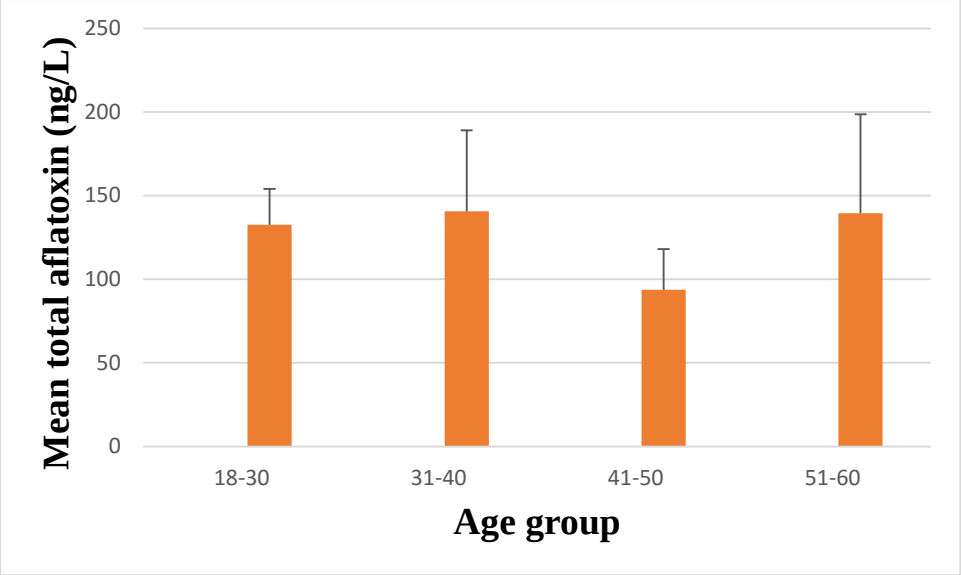

Figure 8. Total aflatoxin concentration (mean ± SE; p = 0.8354) in the sampled population based on the age of the participants.

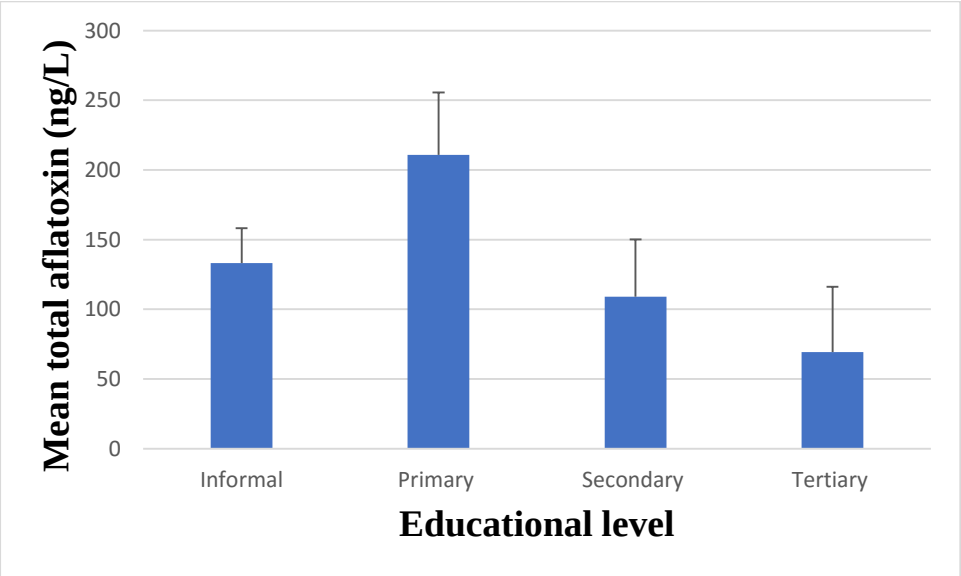

Figure 9. Total aflatoxin concentration (mean ± SE; p = 0.2295) in the sampled population based on the educational level of the participants.

Figure 10. Total aflatoxin concentration (mean ± SE; p = 0.7061) based on the marital status of the participants.

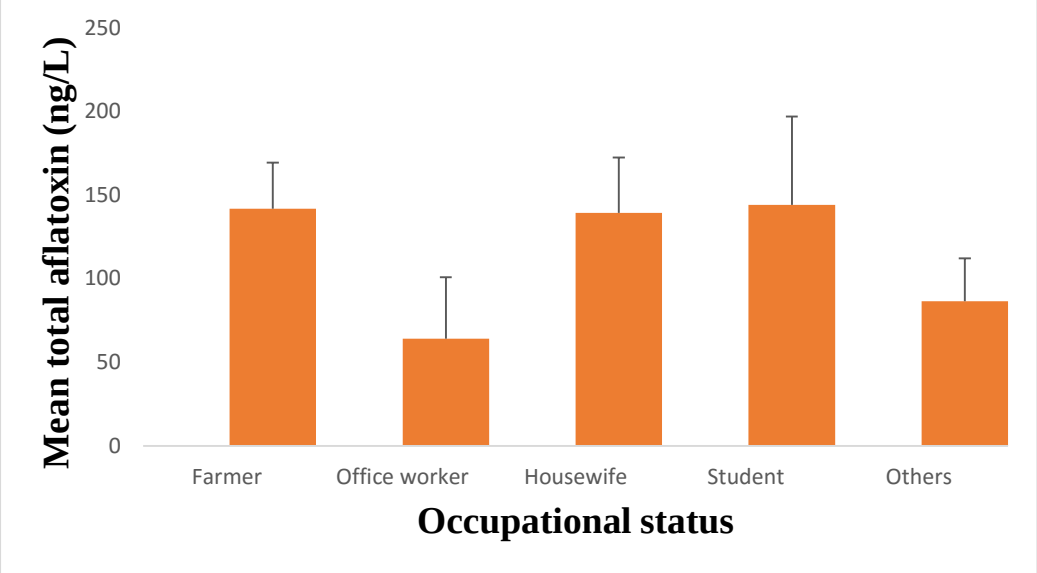

Figure 11. Total aflatoxin concentration (mean ± SE; p = 0.5867) in the sampled population based on the occupational status of the participants.

The mean total aflatoxin concentration in the serum of individuals with a BMI of ≥18.5 kg/m^2 was higher than in individuals with BMI <18.5 kg/m^2, 140.2 and 90.7 ng/L, respectively, but the difference was not statistically significant (p = 0.9033). Figure 12 shows the difference between the mean total aflatoxin concentration of the two BMI groups (≥ 18.5 kg/m^2 and <18.5 kg/m^2). Pearson correlation was performed to examine the correlation between serum

aflatoxin concentration and BMI. The concentration of total serum aflatoxin was not correlated with BMI (r = 0.157 and p = 0.222).

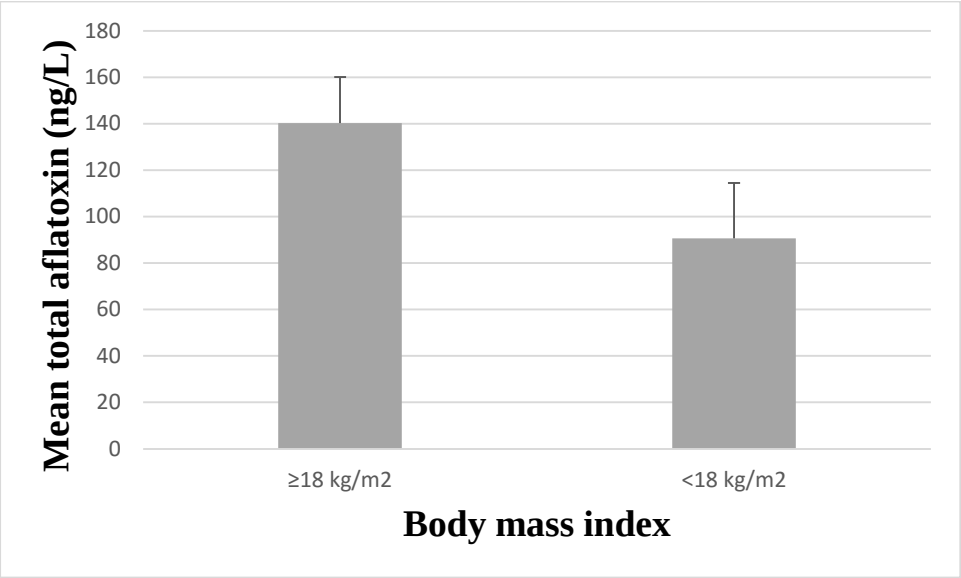

Figure 12. Total aflatoxin concentration (mean ± SE; p = 0.9033) based on the body mass index of the participants.

Table 6 presents the aflatoxin concentrations among the different age groups, gender, education, marital status, occupation, and BMI.

The data on the type and frequency of foods consumed by the study participants were compared with their respective serum aflatoxin concentration. Weekly consumption of different food types; cereals (maize, teff, sorghum, wheat, barley, and millet), pulses (pea, broad bean, haricot bean, chickpea, groundnut, lentil, and soybean), animal products (milk, meat, and egg), spices and alcohol were analysed for their association with participant's serum aflatoxin concentration. According to the analysis, mean total serum aflatoxin was significantly associated with increased consumption of millet (p = 0.031), groundnut (p = 0.019), chickpea (p = 0.023), lentil (p = 0.003) and soybean (p = 0.018). The details of the analysed results are presented in Table 7.

Table 6.Distribution of aflatoxin concentrations in the study population by age, gender, education, marital status, occupation, and body mass index

Factors	**Number of participants**	**Number of positive samples (%)**	**Aflatoxin concentration: mean (ng/L)**					
			AFB_1	AFB_2	AFG_1	AFG_2	AFM_1	AFB_1-lys
Age group								
18–30	62	39 (63%)	42.5	7.8	42.2	57.3	70.2	77.9
31–40	17	12 (71%)	29.0	4.0	36.5	79.3	99.0	54.4
41–50	11	8 (73%)	19.1	3.0	20.4	46.1	26.6	74.7
51–60	10	5 (50%)	104.7	<LOD	<LOD	44.5	73.8	73.4
Gender								
Female	50	30 (60%)	59.2	6.3	46.8	51.7	73.5	61.4
Male	50	34 (68%)	26.0	5.7	31.0	65.6	67.2	78.5
Education								
Informal	46	27 (59%)	40.3	5.5	33.4	53.7	73.4	71.7
Primary	33	22 (67%)	77.0	<LOD	50.5	71.0	70.0	75.8
Secondary	15	10 (67%)	24.5	8.3	24.1	39.0	67.9	54.7
Tertiary	6	5 (83%)	22.9	<LOD	7.9	<LOD	46.4	84.3
Marital status								
Single	19	15 (79%)	27.3	8.3	41.0	54.5	66.5	76.9
Married	81	49 (60%)	47.3	5.5	35.8	59.6	71.3	70.9
Occupation								
Farmer	39	25 (64%)	55.1	3.0	34.7	74.7	68.0	77.8
Office worker	8	6 (75%)	20.5	<LOD	7.9	<LOD	41.8	84.3
Housewife	32	18 (56%)	48.1	6.3	40.7	47.5	77.7	52.4
Student	12	9 (75%)	23.3	<LOD	46.1	69.9	75.7	71.0
Others	9	6 (67%)	34.1	8.3	20.4	8.2	64.5	83.7

BMI								
<18.5 kg/m^2	18	13 (72%)	25.7	7.0	34.9	29.5	50.7	73.5
≥18.5 kg/m^2	82	50 (61%)	46.2	5.8	37.2	65.1	74.7	72.0

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, AFB_1-lys: Aflatoxin B_1-lysine. LOD: Limit of detection. BMI: Body mass index. The means are the average of positive samples.

Table 7.Correlation/association between the consumption frequencies of different food items with total serum aflatoxin concentration of the study participants

Food items	**Mean total aflatoxin concentration (ng/L)**								**p-value**
	Zero consumption	**1/week**	**2/week**	**3/week**	**4/week**	**5/week**	**6/week**	**7/week**	
Maize	157.50	51.85	67.78	218.78	39.19	134.04	204.42	108.33	0.640
Teff	20.59	85.56	130.13	147.35	201.92	166.25	48.64	116.49	0.121
Sorghum	113.18	223.07	169.61	236.89	92.40	220.90	51.85	72.78	0.749
Wheat	104.81	140.81	124.42	228.51	127.19	68.68	350.22	-	0.202
Barely	143.23	130.00	115.53	40.30	350.22	98.33	217.90	-	0.995
Millet	119.78	92.30	268.79	-	462.60	107.80	-	-	0.031*
Pea	114.67	258.52	113.81	92.34	214.57	129.81	64.95	130.00	0.580
Broad bean	113.64	185.21	72.26	123.43	89.50	220.78	135.26	164.96	0.838
Haricot bean	115.05	92.30	202.93	251.99	141.15	-	-	51.85	0.353
Chickpea	121.09	123.57	55.84	156.80	-	427.40	107.80	-	0.023*
Groundnut	116.96	84.50	398.60	94.20	284.28	92.30	-	-	0.019*
Lentil	105.53	125.98	149.51	285.20	392.20	126.21	350.22	-	0.003*
Soybean	120.46	43.60	92.30	204.43	107.80	462.60	-	-	0.018*
Milk	117.81	114.10	144.62	166.67	135.09	259.98	66.23	32.40	0.566
Meat	143.33	112.85	91.51	199.74	83.14	-	-	43.60	0.428
Egg	142.01	151.14	168.04	83.66	66.99	193.41	49.25	43.60	0.353
Spice	-	-	-	-	-	-	-	129.64	-
Alcohol	128.79	154.87	-	-	-	-	-	-	0.495

Consumption frequency of foods per week scored as 0 = do not consume, 1 = once per week, 2 = twice per week, 3 = three times per week, 4 = 4 times per week, 5 = five times per week, 6 = six times per week, 7 = every day. *values significant at 0.05 level

Chapter 5

Discussion

Aflatoxins are secondary metabolites produced by the fungi *A. flavus* and *A. parasiticus.* Exposure to aflatoxins causes hepatocellular carcinoma, immune suppression, child growth impairment, and death. The main route for exposure of humans to aflatoxin is the consumption of aflatoxin-contaminated food. Therefore, the analysis of aflatoxin in the foods consumed by different people in the population helps to estimate the total human aflatoxin exposure. Measuring aflatoxin biomarkers in body fluids is also another method. Considering the bioavailability and toxicokinetics of aflatoxin in the body, the latter is preferred to assess exposure at the individual level (Turner et al., 2012).

Studies from different countries have reported the aflatoxin concentration in human blood (Aydin, Sabuncuoglu, Erkekoglu, Şahin, & Giray, 2014; Kang et al., 2015; Kroker-Lobos et al., 2019; Leong, Rosma, Latiff, & Izzah, 2012), urine (Ali et al., 2016; de Cássia Romero, Ferreira, dos Santos Dias, Calori-Domingues, & da Gloria, 2010; Ezekiel et al., 2014), breast milk (Bogalho et al., 2018; Cantú-Cornelio et al., 2016) and faeces (Mykkänen et al., 2005).

In Ethiopia, a few studies have confirmed the occurrence of aflatoxin in different food commodities (Gizachew et al., 2016; Mohammed et al., 2016; Nigussie, Bekele, Fekadu Gemede, & Zewdu Woldegiorgis, 2018; Taye et al., 2016). To my knowledge, there is no literature evaluating the aflatoxin concentration in the Ethiopian population except Ayelign et al. (2017) who reported 17% of urinary aflatoxin. In the study, the urine samples were collected from children aged 1–4 years from the Tigray and Amhara regions of Ethiopia. The concentrations of AFM_1, AFB_2, AFG_1, and AFG_2 within the ranges of 0.06–0.07, < LOQ–0.06, 0.05–0.06 and 0.06–0.07 ng/mL, respectively were detected in 34/200 (17%) of the sample (Ayelign et al., 2017).

In the present study, AFB_1-lys was the highest concentration detected with a mean of 72.3 ng/L, a range of < LOD–130.0 ng/L and a frequency of 27%. The detection frequency was lower than the results reported by Leong, Rosma, et al. (2012), who detected 97% AFB_1-lys in the serum of adults living in Penang, Malaysia. In the United States, a total of 170 serum samples collected from adults in the Hispanic population living in Bexar County, Texas, AFB_1-lys was detected in 20.6% of the samples within a range of 1.01–16.57 pg/mg albumin (Johnson et al., 2010). In the Kalungu district, Uganda, all serum samples collected from 100

adults were positive for AF-alb adduct ranging from 0 to 237.7 pg/mg albumin (Asiki et al., 2014). A cross-sectional serosurvey conducted using nationally representative serum specimens from the 2007 Kenya AIDS Indicator Survey revealed the presence of AFB_1-lys in 78% of the serum samples within the range of <LOD to 211 pg/mg albumin. The analysed serum samples were from HIV-negative individuals (Yard et al., 2013).

The detection frequency of AFB_1 in present study was 37% > AFG_1 (25%) > AFG_2 (25%) > AFB_2 (5%) with mean concentrations of 43.2, 36.9, 58.6, 6.1 ng/L, respectively. The detection frequency and also the mean concentrations of AFB_1, AFG_1, AFG_2 and AFB_2 in the serum of Ethiopian adults are lower than in the Turkish. Aydin et al. (2014) detected aflatoxin levels in the serum of healthy adult Turkish from the Mediterranean region of Turkey in summer as AFB_1 (87.3%) > AFG_1 (86.5%) > AFG_2 (84.1%) > AFB_2 (76.2%), while the pattern of distribution changed somewhat in winter with AFG_1 (82.2%) > AFB_1 (80.7%) = AFG_2 (80.7%) > AFB_2 (36.3%). Aflatoxin detection frequency from the Black Sea region of Turkey in winter was AFG_1 (85.5%) > AFB_1 (81.4%) > AFG_2 (80.5%) > AFB_2 (43.4%) and in summer was AFB_1 (90%) > AFG_1 (86.4%) = AFG_2 (86.4%) > AFB_2 (65.5%). Regarding the absolute concentrations (total of all aflatoxins) in the two regions of Turkey, the mean serum concentration of total aflatoxin in the population from the Mediterranean region was 0.45 ppb in winter, somewhat less than the equivalent mean detected in summer (0.55 ppb). The mean serum aflatoxin concentration in the study group from the Black Sea region was 0.90 ppb in winter and 1.33 ppb in summer (Aydin et al., 2014). The analytic method used in the Turkey study was HPLC-fluorescence, whereas in the present study, the LC-MS/MS method was used.

In Ethiopia, hepatocellular carcinoma is the eighth most common cancer (Woldeamanuel, Girma, & Teklu, 2013). According to Mekonnen et al. (2017), viral hepatitis, hepatotoxic indigenous drugs and cirrhosis are the major risk factors. Because of its role in the induction of hepatocellular carcinoma, aflatoxin exposure might be a secondary risk factor. In the present study, the highest total aflatoxin concentrations in an individual participant were 465.6 ng/L (male) and 462.4 ng/L (female). All doses of aflatoxin can cause hepatocellular carcinoma, especially in the presence of other major risk factors (hepatotoxic indigenous drugs, cirrhosis, and viral hepatitis) (Mokhles et al., 2007). In most countries, men are at a higher risk of developing hepatocellular carcinoma than women (Bbosa et al., 2013). It follows, therefore, that, in the current study, men testing positive for aflatoxin are likely to be

at higher risk of developing hepatocellular carcinoma than women, in particular when these other risk factors hepatotoxic indigenous drugs, cirrhosis, and viral hepatitis are present.

In the present study, the highest mean total aflatoxin concentration was detected in the samples collected from the Omo Nada district, followed by Manna, Shebe Senbo, Seka Chekorsa, and Kersa, but the differences between districts were not statistically significant. There is no climate data for each district, so it is impossible to determine the differences are due to climatic conditions (e.g. wet/dry, temperature, humidity). This study suggests that Ethiopian adults living in the South-western part of the country are exposed to a relatively higher aflatoxin concentration.

Regarding the socio-demographic factors investigated, there were no significant differences between the mean total aflatoxin concentration and age, gender, education, occupation, or marital status. The result of the current study agrees with the study of Leong, Rosma, et al. (2012), who also reported a non-significant difference between AFB_1-lys and occupation, household number, education level, district and gender of Malay, Chinese and Indian adults living in Penang, Malaysia. A study by Yard et al. (2013) also failed to show an association of AFB_1-lys with age or gender. In my research, the highest mean total aflatoxin was detected in those aged 31–40, which is in agreement with the study of Leong, Rosma, et al. (2012), who showed a higher concentration of AFB_1-lys in study participants aged 31–50. The reason might be because of the relatively higher food intake by those in this age group (Morley, 2001).

With respect to gender, the mean concentration of total serum aflatoxin in males was higher than in females in the current study. Although the detection frequency of total aflatoxin in males was higher than in females for both the Black Sea and the Mediterranean regions in summer and winter in the study from Turkey (Aydin et al., 2014), in summer the mean concentration of total aflatoxin was higher in females than in males for both regions (1.371 ppb in females and 1.288 ppb in males from the Black Sea region and 0.581 ppb in females and 0.469 ppb in males in the Mediterranean region). In winter, the mean total aflatoxin concentration in females was similar to that in males: 0.899 ppb in females and 0.904 ppb in males from the Black Sea region and 0.448 ppb in females and 0.449 ppb in males from the Mediterranean region (Aydin et al., 2014).

In terms of education and occupation, the lowest mean total aflatoxin was detected in persons with tertiary education and office workers. The reason might be better awareness about

aflatoxin toxicity and hence a lower possibility of consuming contaminated food in an educated population. There was no correlation between the mean total aflatoxin concentration and BMI of the participants. However, the mean total aflatoxin concentration in individuals with BMI $\geq$ 18.5 kg/m^2 was higher than individuals with a BMI <18.5 kg/m^2. There is evidence of an increase in the levels of AFB_1 increase plasma free fatty acids, plasma and liver triglyceride (Rotimi et al., 2017), which causes a weight gain, this might be the reason for the higher concentration of aflatoxin in participants with a BMI $\geq$18.5 kg/m^2.

With respect to food frequency data, the mean total aflatoxin concentration was significantly associated with increased consumption of millet, groundnut, chickpea, lentil, and soybean. The results of the present study agree with the study conducted in Malawi by Seetha et al. (2018), who reported a significant association between AFB_1-lys concentration and high groundnut consumption. Unlike the current study, Seetha et al. (2018) measured the concentration of aflatoxins in the groundnut samples as well and detected AFB_1 with a mean concentration of 52.4 µg/kg in 91% of the samples. In the present study, there was no association between maize consumption and the mean total aflatoxin concentration. A study from Guatemala also failed to show an association between maize consumption and AFB_1-albumin concentration in adults (Kroker-Lobos et al., 2019).

Chapter 6
Conclusions

The current study assessed the concentration of AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFB_1-lys in the serum of South-western Ethiopian adults. The most frequently detected serum aflatoxin was AFM_1, and the least was AFB_2. AFB_1-lys exhibited the highest serum concentration among the aflatoxins detected and AFB_2 the lowest. The detection of one or more aflatoxins in the serum of 64% of the study population indicates a high prevalence of aflatoxin accumulation by the South-western Ethiopian adults. Increased consumption of millet, groundnut, chickpea, lentil, and soybean was mostly associated with the high serum aflatoxin concentrations, which suggests contamination of these food types with aflatoxin. Therefore, in Ethiopia, aflatoxin exposure should be considered as a public health problem and different intervention methods, starting from the farm, should be applied. The government should implement rules and regulations on the maximum limit of aflatoxin concentration in food and feed.

This study has limitations, such as small sample size, its five districts do not represent the exposure of the whole Ethiopian population, and the absence of analysis of aflatoxin in participants' food to compare with their serum aflatoxin concentration. The reasons are the high cost of aflatoxin analysis, the logistics of working in collaboration with clinics, and the time limit of my stay in Ethiopia to conduct this study. However, the results I have generated can be used as useful baseline data that contribute to the literature on the exposure of Ethiopian adults to aflatoxin. It would be most useful for others who might like to extend this study in the future. Studies with larger sample sizes, at multiple study areas, and including food analysis could be conducted in the future to get a fuller picture of the Ethiopian population's exposure to aflatoxins.

Chapter 7

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Appendices

Appendix A: Research questionnaire

1. District

- ☐ Manna
- ☐ Seka Chekorsa
- ☐ Shebe Senbo
- ☐ Kersa
- ☐ Omo-Nada

2. Age= _____

- ☐ 18–30
- ☐ 30–40
- ☐ 40–50
- ☐ 50–60

3. Gender

- ☐ Female
- ☐ Male

4. Education

- ☐ Informal education
- ☐ Primary education
- ☐ Secondary education
- ☐ Diploma
- ☐ Bachelor’s degree
- ☐ Master’s degree

5. Marital status

- ☐ Single
- ☐ Married

- ☐ Separated
- ☐ Widowed

6. Occupation

- ☐ Farmer
- ☐ Office worker
- ☐ Student
- ☐ Housewife
- ☐ Other

BMI

Height= _____ m

Weight= _____ kg

BMI= _____ kg/m^2

Food frequency questionnaire

Food item	**Frequency of intake (number of days/week)**							
	Never	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Cereals								
• Maize								
• Teff								
• Sorghum								
• Wheat								
• Barley								
• Millet								
Legumes								
• Pea								
• Broad bean								

• Haricot bean								
• Chickpea								
• Groundnut								
• Lentil								
• Soybean								
Animal source								
• Milk								
• Meat								
• Egg								
Spice								
Alcohol								

Appendix B: Material transfer agreement

Annex V: Material Transfer Agreement

This Material Transfer Agreement (MTA) has been prepared for use by Jimma University and Ghent University in all transfer of research material (samples, derivatives, and specimens) related to the protocol.

Title of the Project: **Assessment of human aflatoxin exposure in Oromia region, Ethiopia using serum and urinary biomarkers**

Provider: Jimma University, Jimma, Ethiopia

Recipient: Ghent University, Belgium

1. Provider agrees to transfer to recipients designated (Dr. Marthe De Boevre and Prof.Dr. Sarah De Saeger) the following research materials (specimen): name of the material/ specimen: **Serum**

Note:

- The research material will only be used for **research purposes** as described in the protocol by recipient's investigator in designated laboratory for the research project described below, under suitable containment conditions.
- This research material will **not be used for commercial purposes** such as screening, production or sale for which a commercialization license may be required.
- **Recipient agrees to comply with all National and International guidelines rules** and regulations applicable to the Research Project and the handling of the Research Material.

a) Are the Research materials of human origin?

Yes [✓] No [ ]

b) If yes, are they collected according to the details in the protocol and in adherence to National Health Research Ethics Review Committee (NERC) and the University of Lincoln and Jimma Ethics Review Committee recommendations and their approval?

Yes [✓] No [ ]

This research material and its derivatives will be used by recipient's investigator solely in connection with the following research project ("Research Project")described with specificity as follows: **Assessment of human aflatoxin exposure in Oromia region, Ethiopia using serum and urinary biomarkers.**

2. In all presentations or written publications concerning the research project, recipient will seek agreement of provider and acknowledge provider's contribution of this research material unless requested otherwise.
3. This research material represents a significant contribution on the part of provider and is considered proprietary to provider. Recipient therefore agrees to retain control over this research Material and further agrees not to transfer the research Material to other people not under her/his direct supervision without advance written approval of provider. The research material will be disposed of as agreed upon per protocol at the end of analysis completion of the project.
4. The provider does not take any responsibility for loss, damage, wastage or spoilage of the research material during or after shipment to the address provided by the Recipient under conditions agreed to in the protocol on shipment of the samples. This Research Material is provided as a service to the research community. *IT IS BEING SUPPLIED TO RECIPIANT WITH NO WARRANTIES, EXPRESS OR IMPLIED, INCLUDING ANY WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE*. Provider makes no representations that the use of the research material will not infringe any patent or proprietary right of third parties.
5. The recipient shall notify the provider in witting of any intention, improvement, modification discovery or development to the material or the information made by Recipient or parties, collaborating with Recipient, here in after referred to as "invention". Nothing in this agreement shall, however, be construed as conveying to the provider any rights under any patents or other intellectual property to such invention, other than as explicitly provided herein. At its option the provider shall be entitled to receive sample of any materials derived from the Materials for its own research and evaluation purposes only.
6. The under- signed provider and Recipient expressly certify and affirm that the contents of any statements made herein are truthful and accurate.
7. Any additional terms (use an attached page if necessary):
8. The provider maintains, ownership right of the research material and its derivatives unless stated otherwise. The provider will retain a copy (aliquot) of every sample sent abroad as much as possible for local research needs.

Material Transfer Agreement Signature page

For Recipient:

Recipient's Investigator

Duly Authorized

Dr. Marthe De Boevre & Prof. Dr. Sarah De Saeger

Signature / Stamp

LABORATORY OF FOOD ANALYSIS
DEPARTMENT OF BIOANALYSIS
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Lab. of Food Analysis

S. De Saeger

DE BOEVRE MARTHE

Date 12/11/2018

Date 12/11/18

Mailing Address for Material:

Mailing Address for Notices:

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Institute of Health Jimma University

For Provider

Provider's Investigator

Name Prof. Tefera Belachew

Signature

Date 25/10/2018

Mailing Address:
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Duly Authorized

Date

Mailing Address for Notices:
P.o.Box
Tel:
Fax:

Zelalem Mengesha (PhD)
Associate Professor, Health
Research and Postgraduate
Director

Institute of Health
Jimma University

Appendix C: Ethical clearance letter from Lincoln University

Research Management Office

T 64 3 423 0817

PO Box 85084, Lincoln University

Lincoln 7647, Christchurch

New Zealand

www.lincoln.ac.nz

28 August 2018

Application No: 2018- 27

Title: Assessment of human aflatoxin exposure in Oromia Region, Ethiopia, using serum and urinary biomarkers

Applicant: L Terefe

The Lincoln University Human Ethics Committee has reviewed the above noted application.

Thank you for your response to the questions which were forwarded to you on the Committee's behalf.

I am satisfied on the Committee's behalf that the issues of concern have been satisfactorily addressed. I am pleased to give final approval to your project.

Please note that this approval is valid for three years from today's date at which time you will need to reapply for renewal.

Once your field work has finished can you please advise the Human Ethics Secretary, Alison Hind, and confirm that you have complied with the terms of the ethical approval.

May I, on behalf of the Committee, wish you success in your research.

Yours sincerely

Grant Tavinor

Chair, Human Ethics Committee

PLEASE NOTE: The Human Ethics Committee has an audit process in place for applications. Please see 7.3 of the Human Ethics Committee Operating Procedures (ACHE) in the Lincoln University Policies and Procedures Manual for more information.

Appendix D: Ethical clearance letter from Jimma University, Ethiopia

JIMMA UNIVERSITY
ጅማ ዩኒቨርሲቲ

ቁጥር
Ref.No JHRPGD/687/19
ቀን
Date 29 ሚያዝ, 2019

Institutional Review Board (IRB)
Institute of Health
Jimma University
Tel : +251471120945
E-mail : zeleke.mekonnen@ju.edu.et

To: Mrs. Lemlem Arega

Subject: Ethical approval of research protocol

The IRB of institute of health has reviewed your research project entitled:

"Assessment of human aflatoxin exposure in Oromia region, Ethiopia using serum and urine biomarker"

This is to notify that this research protocol as presented to the IRB meets the ethical and scientific standards outlined in national and international guidelines. Hence, we are pleased to inform you that your protocol is ethically cleared.

We strongly recommended that any significant deviation from the methodological details indicated in the approved protocol must be communicated to the IRB before they are implemented.

With regards!

Zeleke Mekonnen (PhD)
Associate Professor, Health
Research and Postgraduate
Director

Appendix B: Consent form in English

Name of the Project: Human aflatoxin exposure in south-western Ethiopia assessed using serum biomarkers

This consent form is related to the interview, measuring of weight and height and sampling of blood that is about to take place.

As a participant, you will be asked questions regarding your sociodemographic characteristics (age, gender, education, etc) and types of food you consume weekly. Questions for sociodemographic characteristics and list of food items are provided on the questionnaire. After the interview, measuring your weight and height; a collection of blood sample will be carried out.

I have read and understood the objective of the research. I agree to participate in the project, and I give my permission for the publication of the results of the project with the understanding of the privacy of the data. I also understand that I can withdraw from the study at any stage.

Name: _____

Signature: _____ Date: _____

Appendix C: Consent form in Amharic language

<u>የፕሮጀክቱ ስም፦ በኢትዮጲያ ኦሮሚያ ክልል የሰረማ ዩሪነሪ ባዮማርከርን በመጠቀም ሰዎች ላይ ያለውን የአፍላቶክሲን ተጋላጭነት ማማማም</u>

ይህ የስምምነት ፎርምከቃለመጠይቁ ጋር የሚገናኝ ሲሆን የክብደት እና ቁመት ልኬት እንዲሁምየደም ሽንት ናሙና ይወሰደበታል።

እንደጥናቱ ተሳታፊ ዕድሜዎን፣ ጾታዎን፣ የትምህርት ደረጃዎን እና የመሳሰሉትን ማህበራዊ መረጃዎችዎን እንዲሁም በሳምንት ውስጥየሚመገቡትን የምግብ ዓይነት ይጠየቃሉ። የማህበራዊ መረጃዎችን የተመለከቱ ጥያቄዎችና የምግብ ዓይነት ማዘርዝሮች በመጠይቁ ላይ ሰፍረዋል። ከቃለመጠይቁ በኋላ የክብደትና ቁመት ልኬት ይወሰዳል። እንዲሁም የደም ሽንት ናሙና ይሰበሰባል።

የጥናቱ ዓላማአንብቤ ተረድቻለሁ፣ በፕሮጀክቱ ላይ ለመሳተፍ ተስማምቻለሁ። በተጨማሪምለጥናቱ ውጤት ህትመት የመረጃውን ጭምጥነፃነት በማሟ ፍቃደኛ መሆን ን አሳውቃለው፡ ከዚህምበላይ በማንኛውምጊዜ ከጥናቱ እራሴን ማግለል እንደምችል አውቃለው፡

ስም___________________

ፊርማ_________________ ቀን __________________

Appendix D: Consent form in Afaan Oromoo language

Maqaa proojaktichaa: Biyya Itiyoopiyaa, naannoo oroomiyaattii seeramifi yurinarii baayoomaarkarii fayadamuun aflaatooksinni namoota irra mudatuu xinxaluu

Formiin waligaltee kun waraqaa gaaffi wajjin kan dhiyaata yoo ta`ee safari mizanaafi hojjaa akkasumas fakeenyi dhigaafi fincaanii ni fudhatamaa.

Akka qooranichaatti umuriin saalli, sadarkaa barnootnii kessaniifi kan kana fakaatan nigaafatamtu. Akkasumas torbee tokko kessatti gosa nyaataa fayadamtan ni gaafatamtuu. Gaafileewwan kana ilaalataniifii gosootni nyaataa gadifageanyaan waraqaa gaaffiiratti safaraniruu. Waraqaan gaaffi erga guutamee booda safari mizaanaf hojjaa, fakeenyi dhigaafi fincaanii ni sasaabamaa.

Yaada qoranichaa dubisee hubadheeraa. Piroojakticharatti hirmaachuuf waligaleeraa. Kanaratti, maxxanfamuun qoranoo kanaa eyama koo akka ta` ee nan ibsaa. Akkasamus yeeroo barbaadameetti qoranicharaa ofhambisuu akkan danda`uu nan beeka.

Maqaa _____

Mallattoo _____ Guyya _____

Appendix E: Pictures captured during sample and data collection

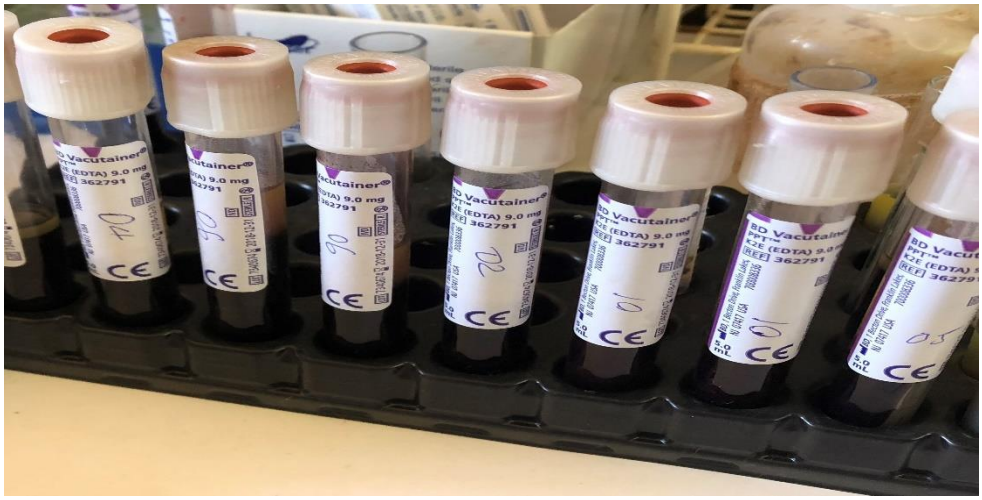
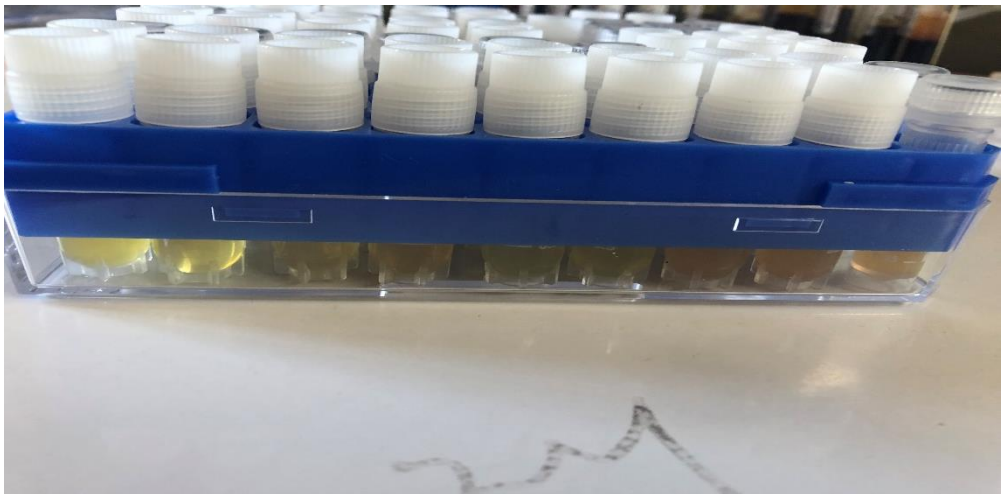


Figure H1. Collected blood samples before centrifugation.



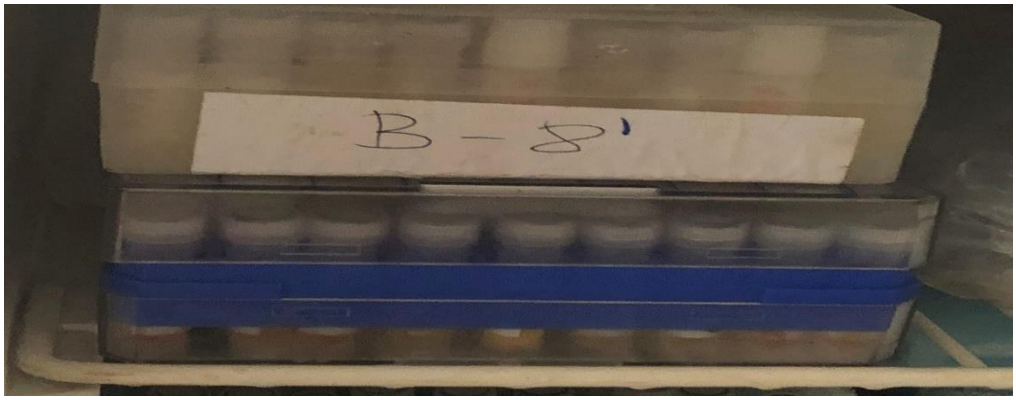


Figure H2. Serum samples.

Figure H3. Health centres where collection of samples and data took place.

Appendix F: Statistical analysis

Analysis of Variance Table for TOTAL AF

Source	DF	SS	MS	F	P
DISTRICT	4	145263	36315.8	1.51	0.2107
Error	57	1368584	24010.2		
Total	61				

Note: SS are marginal (type III) sums of squares

Grand Mean 136.97 CV 113.13

Statistix 8.0
16:19:48

Analysis of Variance Table for TOTAL AF

Source	DF	SS	MS	F	P
AGE	3	22056	7351.9	0.29	0.8354
Error	58	1491792	25720.5		
Total	61				

Note: SS are marginal (type III) sums of squares

Grand Mean 130.92 CV 122.50

Statistix 8.0
16:20:31

Analysis of Variance Table for TOTAL AF

Source	DF	SS	MS	F	P
GENDER	1	4886	4886.4	0.19	0.6609
Error	60	1508961	25149.3		
Total	61				

Note: SS are marginal (type III) sums of squares

Grand Mean 140.85 CV 112.59

Statistix 8.0
16:21:13

Analysis of Variance Table for TOTAL AF

Source	DF	SS	MS	F	P
EDUCATION	3	107622	35873.9	1.48	0.2295
Error	58	1406226	24245.3		
Total	61				

Note: SS are marginal (type III) sums of squares

Grand Mean 118.37 CV 131.54

Statistix 8.0
16:21:51

Analysis of Variance Table for TOTAL AF

Source	DF	SS	MS	F	P
MARITAL	1	3614	3613.5	0.14	0.7061
Error	60	1510234	25170.6		
Total	61				

Note: SS are marginal (type III) sums of squares

Grand Mean 135.56 CV 117.04

Statistix 8.0
16:22:37

Analysis of Variance Table for TOTAL AF

Source	DF	SS	MS	F	P
OCCUPATION	4	72097	18024.3	0.71	0.5867
Error	57	1441750	25293.9		
Total	61				

Note: SS are marginal (type III) sums of squares

Grand Mean 122.44 CV 129.89

Statistix 8.0
16:23:34

Analysis of Variance Table for TOTAL AF

Source	DF	SS	MS	F	P
BMI	8	90440	11305.0	0.42	0.9033
Error	53	1423408	26856.7		
Total 61					

Note: SS are marginal (type III) sums of squares

Grand Mean 127.14 CV 128.90