ADDIS ABABA UNIVERSITY
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AFLATOXIN AND MICROBIAL CONTAMINATION OF COMPLEMENTARY FOODS AND EXPOSURE ASSESSMENT AMONG YOUNG CHILDREN USING URINARY AFLATOXIN BIOMARKERS IN ETHIOPIA

A PhD Dissertation in Food Science and Nutrition

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To the Almighty God and His Mother

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LIST OF ABBREVIATIONS/ACRONYMS

AAU	Addis Ababa University
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFM1	Aflatoxin M1
AFM2	Aflatoxin M2
AFQ1	Aflatoxin Q1
ANOVA	Analysis of Variance
BGB	Brilliant Green Bile
BGLB	Brilliant Green Lactose Bile
BPW	Buffered Peptone Water
CAC	Codex Alimentarius Commission
CCPs	Critical Control Points
CDC	Centers for Disease Control and Prevention
CFs	Complementary Foods
CFU	Colony Forming Unit
CRD	Completely Randomized Design
CSA	Central Statistical Agency
CV	Coefficient of Variation
DNA	Deoxyribonucleic Acid
EAC	East African Community
ELISA	Enzyme Linked Immunosorbent Assay
ENA	Emergency Nutrition Assessment
EOC	Ethiopian Orthodox Church
ESI	Electrospray Ionization
ESIA	Enterobacter Sakazakki Isolation Agar
FAO	Food and Agriculture Organization of the United Nations

FDA	Food and Drug Administration
FFQ	Food Frequency Questionnaire
FSMS	Food Safety Management Systems
GAP	Good Agricultural practices
GBs	Grain Banks
GDP	Gross Domestic Product
GMP	Good Manufacturing Practices
HACCP	Hazard Analysis of Critical Control Point
HAZ	Height-for-Age Z-score
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HEWs	Health Extension workers
HHs	Households
IARC	International Agency for Research on Cancer
ICH	International Conference on Harmonization
IITA	International Institute of Tropical Agriculture
IMViC	Indole, Methyl red, Voges-Proskauer, Citrate
ISO/TS	International Organization for Standardization/Technical Specification
KEBS	Kenya Bureau of Standards
L-EMB	Levine's Eosin-Methylene Blue
LOD	Limit of Detection
LOQ	Limit of Quantification
LST	Lauryl Tryptose
MI	Micronutrient Initiative
MLST	Modified Lauryal Sulphate Tryptose broth
MMC	Matrix Matched Calibration
MPN	Most Probable Number
MUAC	Mid Upper Arm Circumference
NRERC	National Research Ethics Review Committee
PCA	Plate Count Agar

PIF	Powdered Infant Formula
RiPPLE	Research–inspired Policy and Practice Learning in Ethiopia
RNA	Ribonucleic Acid
RSD_r	Intraday Precision
RSD_R	Intermediate Precision
S/N	Signal-to-noise ratio
SNNP	Southern Nations Nationalities and Peoples
SNNPR	Southern Nations Nationalities and Peoples Region
SOPs	Standard Operating Procedures
SPSS	Statistical Product and Service Solutions
SSA	Sub-Saharan Africa
SSE	Signal Suppression/Enhancement
TSA	Tryptone Soya Agar
VP	Voges-Proskauer
WAZ	Weight-for- Age Z-score
WHO	World Health Organization
WHZ	Weight-for-Height Z-score

ABSTRACT

With 40% of children five years and under being stunted (CSA, 2014), Ethiopia has one of the highest rates of under nutrition in Sub-Saharan Africa. Growth faltering reaches its peak, during the period when complementary foods (CFs) are often introduced, indicating that inappropriate introduction and patterns of complementary feeding may contribute to the problem. Interventions have been designed to improve the quality of CFs, but these have paid little attention to the potential contamination with aflatoxin and microbial pathogens. Therefore, this study intended to assess the knowledge and practices of the mothers/caregivers on issues related to aflatoxin in CFs, investigate the safety of CFs in terms of microbial and aflatoxin contamination, assess aflatoxin exposure among young children using urinary biomarkers and develop HACCP based SOPs for the safe preparation of CFs.

The knowledge and practice study involved 195 mothers from 20 Districts from Amhara, Tigray, Oromia, and Southern Nations Nationalities and Peoples (SNNP) regions and addressed a range of issues related to aflatoxin in CFs using structured questionnaires. A total of 146 samples collected from 20 Districts were tested for the presence of *Cronobacter sakazakii (C. sakazakii)*, coliforms and *Escherichia coli (E. coli)*; and determined for the levels of total aflatoxin. The incidence of *C. sakazakii* was detected using ISO/TS 22964:2006 method while coliforms and *E. coli* were detected using the conventional most probable number (MPN) method. The levels of total aflatoxins were determined using Enzyme linked immunosorbent assay (ELISA). The biomarker study was conducted with 200 urine samples collected from 200 children and assessed for the levels of AFB1, AFB2, AFG1, AFG2 and AFM1 using a validated LC-MS/MS method.

The knowledge and practice study results indicate that, 95% (186/195) of the respondents ploughing the land before growing the next crop, 91% (177/195) of them used crop rotation schedule, and 81% (157/195) reported the practice of removing old seed heads, stalks and other debris. A total of 70% (138/195) respondents used the threshing method known as trampling by hoofed animals on a threshing bare floor. Among the respondents, 27% (53/195) of them used ‘Gota’, 28% (54/195) used both ‘Gota’, ‘Gotera’ and polypropylene bags for home storage of cereals and legumes. Only, 7% (14/195) of the respondents used underground pit storage.

C. sakazakii was detected in 12% (17/146) of the samples, while 45% (66/146) and 6% (9/146) of the samples collected were positive for coliforms and *E. coli*, at a level of >1100 CFU/g and 150 CFU/g respectively. Total aflatoxin was detected in19 out of 20 (with mean range of 2.3-88 µg/kg), 62 out of 66 (with mean range of 0.3-9.9 µg/kg), and 59 out of 60 (with mean range of 0.5-12.4 µg/kg) moldy, pre-milling and CFs samples respectively. The biomarker study also revealed that, aflatoxins were detected in 17% (34/200) of the urine samples whereby four out of five analyzed aflatoxins were detected. AFM1 was detected in 7% (14/200) of the urine samples in a range of 0.06-0.07 ng/mL. AFB2, AFG2 and AFG1 were detected in respectively 4.5% (9/200), 3% (6/200) and 2.5% (5/200) of the urine samples whereas AFB1 was not detected in any of the samples. In this study, there was no correlation between the different malnutrition categories (stunted, wasting and underweight) and aflatoxin exposure.

In conclusion, most respondents were unaware of toxic effects of aflatoxins on human and animal health. The identification of more microbial contamination in CFs from post production to following one month storage implies poor hygienic practices or cross-contamination by production equipment. Although aflatoxin levels were considered safe for consumption in most samples, more effort should be implemented to reduce these contamination level, particularly as these CFs are intended for direct consumption by young children. The biomarker analysis showed a clear exposure of young children to aflatoxins. Therefore, message to improve public awareness is important to prevent the health consequences of aflatoxins. Further, implementation of the HACCP based SOPs should be encouraged for preventing the CFs from the risks of aflatoxin and microbial contamination.

Keywords: Complementary food; Mothers; Aflatoxin; *C. sakazakii*; Coliforms; *E. coli*, Grain banks; Households; Biomarker; Urine; Children; Exposure assessment

CHAPTER 1. GENERAL INTRODUCTION

1.1 Background

Globally, 159 million and in Africa, 58 million children under 5 years of age are stunted (UNICEF, 2015). Many school-age children, adolescents and adults today suffer the consequences of the stunting caused during their early years of life (Eggersdorfer *et al*., 2013). In Ethiopia, 40% children younger than 5 years are stunted, which is one of the highest rates of under nutrition in Sub-Saharan Africa (CSA, 2014). Growth faltering reaches its peak between 6-23 months of age, during the period when complementary foods (CFs) are often introduced (Brown *et al*., 1998), indicating that inappropriate introduction and patterns of complementary feeding may contribute to the problem. Total losses in productivity due to stunting for 2009 are estimated at approximately 53.6 billion Ethiopian Birr, which is equivalent to 16% of Ethiopia's gross domestic product (GDP) (FMoH, 2013). UNICEF Ethiopia and partners, recognizing this problem, developed a project for the local production of CFs using locally available ingredients that would optimize the nutritional intake, and thus nutritional status, of children 6-23 months in rural and semi-urban settings of Amhara, Tigray, Oromia and Southern Nations Nationalities and Peoples (SNNP) regions.

The objective of this project was to develop and promote community based production of CFs as an efficient means to address the existing problems in complementary feeding practices. In this context, two different models were designed for the implementation of a community based production of CFs: the rural and semi-urban models (settings).

The rural model is organizing the community to initiate and implement a centralized production and distribution of CFs through a bartering system. In this model, the project provides limited equipment (storage bins, roasting pans, bags, balance and sealer) and most of the raw products (cereals/legumes) are contributed by the community, depending on their availability. These are stored in a grain bank (GB), a space provided by the community. Women are identified and trained to process the CFs by following a standard formulation developed by the Center for Food Science and Nutrition, AAU. The bartering aspect of this model invited mothers to bring 2 kg of raw materials and receives 3 kg of prepared CFs in return.

The semi-urban model is similar to that of the rural model, in that the CFs are prepared by locally trained women, and the community provides a building to store equipment needed for the processing. However, the objective of this model is to allow the women to sell the product at a reasonable profit. Therefore, the supplies provided by the project are much more extensive than in the rural model (mills, sieve for cleaning the raw product, mixer to ensure complete mixture of the cereal/legume product, sealer for bagged product, scales for ingredients and final product, and electrical accessories). The project also provides the initial stock of cereals and legumes. The women's group identified to manage the project receives training on cleaning and proper handling, production of CFs and storage of food items and equipment.

Production and distribution through both the rural and semi-urban models have been well integrated and accepted by the communities involved in the pilot. However, the CFs and its ingredients have greater chance of being contaminated with aflatoxins, and microbial agents such as *Cronobacter Sakazakii* (*C. sakazakii*), coliforms, *Escherichia coli* (*E. coli*), and other foodborne pathogens.

Aflatoxins are toxic secondary metabolites that are produced mainly by filamentous fungi as a by-product of their metabolism and produced on a wide range of matrices (Calvo *et al*., 2002; Pildain *et al*., 2008). Aflatoxins contaminate many food crops, but cereals and groundnuts are the most susceptible (Reddy *et al*., 2010; Wild & Gong, 2010; Yard *et al*., 2013). Contamination of cereals and cereal products by fungi and aflatoxins results in quality and nutritional losses and represents a considerable threat to the food chain (Magan & Aldred, 2007).

Aflatoxins can have both acute and chronic effects to human health when ingested, inhaled, or absorbed through skin (Bennett & Klich, 2003; Boonen *et al*., 2012; Reddy *et al*., 2010). Chronic exposure especially during child hood could be linked to the early onset of hepatocellular carcinoma (HCC) (Gong *et al*., 2016; Kirk *et al*., 2005; Polychronaki *et al*., 2008; Wild & Gong, 2010; Zain, 2011). Aflatoxin exposure has also been associated with growth faltering (Gong *et al*., 2002; Gong *et al*., 2004; Khlangwiset *et al*., 2011; Turner, 2013, Turner *et al*., 2007; Wild *et al*., 2015) and immune suppression in young children (Turner *et al*., 2003).

Aflatoxins impaired growth by targeting the intestinal tract, which may promote intestinal damage, resulting in reduced absorption capacity of essential nutrients and impaired intestinal barrier function (Smith *et al*., 2012). Further, young children are more vulnerable to aflatoxin exposure. This is mostly because of a lower detoxification capacity, rapid growth, and high intake of food per kg of body weight (Lombard, 2014). Acute exposure known as aflatoxicosis can lead to vomiting, abdominal pain and liver failure (Zain, 2011), with documented fatality rates of 39% reported in Kenya in 2004 (Probst *et al*., 2007).

Previous findings reported high aflatoxin content in certain cereals (wheat, maize, teff, sorghum and finger millet), that are the most common ingredients for the production of CFs in Ethiopia. A study by Chala *et al*. (2014) reported high aflatoxin content in sorghum and finger millet. Another study by Ayalew *et al*. (2006) reported the presence of aflatoxin in barley, sorghum, teff, and wheat samples. A similar study by Ayalew (2010) also reported aflatoxin in maize samples. So far, there is no any study in Ethiopia that reported the levels of aflatoxin in legumes (broad bean, pea, chick pea, and haricot bean), commonly used in the production of CFs. Hence, the extent of these problems in the two community-based CFs production centers needs to be evaluated. In addition, hazard analysis critical control point (HACCP) based standard operating procedures (SOPs) that will eventually prevent such contaminations are also required.

Similarly, the CFs might have the tendency to be contaminated by C. *sakazakii*, coliforms, and *E. coli* that cause undernutrition and health challenges in young children. *C. sakazakii* has been implicated as a cause of bacterial infections in infants since 1961 (Bowen & Braden, 2006). *C. sakazakii* infections cause meningitis and necrotizing enterocolitis. Even though the incidence is low, this pathogen has a 40–80% mortality rate in infected infants (Forsythe, 2005).

Foodborne microbial agents; coliforms and *E. coli* may cause diarrhoeal diseases and ill-health in young children (Motarjemi, 2000). Globally, diarrhoea is the second leading cause of death for children under the age of five (Black *et al*., 2003) and is a leading cause of growth faltering and malnutrition. A pronounced proportion of diarrhoeal incidences occur due to foodborne pathogens transmitted by unhygienic preparation of foods in households (Sheth & Dwivedi, 2006). In

Ethiopia, dehydration from diarrhoea is a major cause of death in infancy and childhood and 13% of children under age 5 were reported to have had diarrhoea (CSA, 2012).

Therefore, evaluating the safety of the CFs produced at community level using locally available ingredients in terms of aflatoxins, *C. sakazakii*, coliforms and *E. coli* are of paramount importance to safeguard the health of young children in the study areas. Besides, it is extremely important to provide evidence for safety interventions and regulations.

However, evaluating the levels of aflatoxin in the CFs is not enough to assess the levels of aflatoxin exposure among young children. Since there are other routes through which the young children can be exposed to aflatoxins: breast feeding (Polychronaki *et al*., 2007); physical contact through the skin and inhalation (Boonen *et al*., 2012; Reddy *et al*., 2010). In addition, there are variations among individuals in terms of absorption, distribution, metabolism and excretion of the toxins (Arcella & Leclercq, 2004).

As a result, biomarkers in biological fluids such as blood and urine have the possibility to be quantitative dosimeters of individual exposure, as well as initial cautioning signs of health effects (Turner *et al*., 2000; Wild & Gong, 2010). Urinary aflatoxin biomarkers of exposure and effect for aflatoxins have been validated in comprehensive studies in animals (Song *et al*., 2013) and humans (Abia *et al*., 2013; Ediage *et al*., 2012; Ediage *et al*., 2013; Ezekiel *et al*., 2014; Mykkane *et al*., 2005; Piekkola *et al*., 2012). The use of urinary aflatoxin as biomarkers reflects not only the dietary exposure of the individual but also the uptake, toxicokinetics and toxicodynamics (Ediage *et al*., 2012; Polychronaki *et al*., 2008).

Aflatoxin B1 (AFB1) and its metabolites are excreted through urinary routes (Abia *et al*., 2013; Ali *et al*., 2015; Ediage *et al*., 2013; Ezekiel *et al*., 2014; Mykkanen *et al*., 2005; Piekkola *et al*., 2012). The aflatoxin metabolite, aflatoxin M1 (AFM1), is also excreted in breast milk if the mother is exposed to aflatoxin contaminated foods (Adejumo *et al*., 2013; Atasever *et al*., 2014; Iha *et al*., 2014; Polychronaki *et al*., 2006; Polychronaki *et al*., 2007). Besides, aflatoxin-albumin adduct is usually the target metabolite in blood for aflatoxin exposure (Anitha *et al*., 2014; Jolly *et al*., 2015; Schleicher *et al*., 2013; Sharma & Farmer, 2004; Tang *et al*., 2008; Watson *et al*., 2016). However, obtaining blood samples from younger children creates a high barrier for participation. As a result, urine was chosen as a target matrix for this study.

Therefore, in addition to evaluating the levels of total aflatoxin in the CFs, assessing aflatoxin exposure among young children using urinary biomarkers are important to get new insights in the issue of aflatoxin and to implement targeted prevention and control of aflatoxin exposure.

1.2 Statement of the problem

In Ethiopia children jump from their mother's breastmilk to adult food and CF is neither understood nor utilized in rural communities. Children therefore rarely get energy and micronutrient dense food hence the need for the project. The community based production and distribution of the CFs using locally available ingredients in all the regions have been well integrated and accepted by the communities. However, the CFs and their local ingredients were in most cases contaminated by mold, aflatoxin, *C. sakazakii*, coliforms, *E. coli*, and other foodborne pathogens because of sub-standard postharvest management and food storage and lack of any preventive measures to reduce this risk.

Previous studies also reported high aflatoxin content in certain cereals (maize, wheat, teff, sorghum, finger millet and barley) that are potentially used for the production of CFs (Ayalew *et al*., 2006; Ayalew, 2010; Chala *et al*., 2014). Chronic aflatoxin exposure has been linked to HCC (Gong *et al*., 2016; Kirk *et al*., 2005; Omer *et al*., 2004; Polychronaki *et al*., 2008; Wang *et al*., 2001; Wild & Gong, 2010) and to growth faltering in children (Gong *et al*., 2002; Gong *et al*., 2004; IARC, 2016; Polychronaki *et al*., 2008; Turner *et al*., 2007; Turner, 2013; Wild *et al*., 2015). Aflatoxins also impair immunity in children (Gong *et al*., 2016; Jiang *et al*., 2005; Turner *et al*., 2003). Similarly, foodborne microbial agents such as *C. sakazakii* infections cause meningitis and necrotizing enterocolitis (Forsythe, 2005). Coliforms and *E. coli* may cause diarrhoeal diseases and ill-health in young children (Motarjemi, 2000). Globally, diarrhoea is the second leading cause of death for children under the age of five (Black *et al*., 2003) and is a leading cause of growth faltering and malnutrition. In Ethiopia, dehydration from diarrhoea is a major cause of death in infancy and childhood (CSA, 2012).

Further, evaluating the levels of aflatoxin from the CFs and its ingredients is not enough to assess aflatoxin exposure among young children. Since, aflatoxin contamination in food and food commodities is heterogeneous with marked seasonal variations (Turner *et al.*, 2012; Williams *et al.*, 2004). Second, the young children may be exposed to aflatoxins through other routes like physical contact via the skin and inhalation (Boonen *et al.*, 2012). Finally, there are individual variations in terms of absorption, distribution, metabolism and excretion of aflatoxins (Arcella & Leclercq, 2004). Hence, urinary biomarkers should be used to sidestep the above-mentioned limitations. Therefore, investigating the safety of CFs in terms of microbial and aflatoxin contamination and assess aflatoxin exposure among young children using urinary biomarkers are very timely to know the status of the problem and to implement targeted prevention and control of aflatoxin exposure.

1.3 Research questions

- Do mothers have the knowledge and practices of preventing the CFs and its ingredients from the risks of mold and aflatoxin contamination?
- What is the status of the CFs and their ingredients in terms of *C. sakazakii*, coliforms and *E. coli* contamination?
- Are the CFs and their ingredients contaminated by aflatoxin? Are the levels of aflatoxin surpassing the maximum limits?
- Are the young children in the study area exposed to aflatoxin? Which urinary aflatoxin is frequently detected?
- Is it possible to develop and implement HACCP based SOPs to prevent the CFs from mold, aflatoxin and microbial contamination?

1.4 Objective

1.4.1 General objective

To investigate the safety of CFs in terms of microbial and aflatoxin contamination and assess aflatoxin exposure among young children using urinary biomarkers and develop HACCP based SOPs.

1.4.2 Specific objectives

- Assess the knowledge and practice of mothers/caregivers of young children to prevent the CFs from mold and aflatoxin contamination,
- Detect the presence of *C. sakazakii* and determine the contents of coliforms and *E. coli* in moldy (visually) cereals and legumes, pre-milling cereals and legumes and CFs,
- Determine the contents of aflatoxin in moldy cereals and legumes, pre-milling cereals and legumes and CFs to see the occurrence and levels of contamination,
- Assess aflatoxin exposure among young children using urinary biomarkers,
- Develop HACCP based SOPs to prevent the CFs from mold, aflatoxin and microbial contamination.

CHAPTER 2. LITERATURE REVIEW

2.1 Complementary food

Adequate nutrition during infancy and early childhood is essential to the development of every child’s full human potential. It is well recognized that the period from birth to two years of age is a “critical window” for the promotion of optimal growth, health and behavioural development. Further, this is the peak age for growth faltering, ocurrences of hidden hunger, and common childhood illnesses such as diarrhoea. After a child reaches two years of age, it is very difficult to reverse stunting that has occurred earlier (Martorell *et al*., 1994; Shrimpton *et al*., 2001).

The immediate consequences of undernutrition during these determinative years include significant morbidity and mortality and delayed cognitive and psycho motor development. In the long term, malnutrition in early lives are linked to impairments in intellectual performance, work capacity, reproductive outcomes and overall health in later years. Thus, the cycle of malnutrition continues, as the malnourished girl child faces greater odds of giving birth to a malnourished, low-birth-weight infant when she grows up. Poor infant feeding practices, coupled with high rates of infectious diseases, are the main causes of malnutrition during the first two years of life (WHO, 2005).

As a result, WHO recommends the introduction of solid food to infants starting six months of age because by that age breast milk alone is no longer adequate to maintain a child’s optimal growth. Consuming or giving infants other foods or fluids than breast milk is considered the period of complementary feeding between 6 and 23 months of age (Brown *et al*., 1998). This 18 months’ interval is the largest part of the ‘‘1000 days’’ encompassing pregnancy and the first 2 years after birth, now viewed as the key window of opportunity for preventing under nutrition and its long-term adverse consequences (Stewart *et al*., 2013).

CF is any food whether manufactured or locally prepared, suitable as a complement to breast milk or to infant formula. CFs can be especially prepared for the infant or can be the same foods available for family members, modified in order to meet the eating skills and needs of the infant (Monte *et al*., 2004). As much as possible CFs are rich in energy and in micronutrients (especially iron, zinc, calcium, vitamin A, vitamin C and folates), free of contamination, without much salt or spices, easy to eat and easily accepted by the infant, in an appropriate amount, easy to prepare from family foods, and at a cost that is acceptable by most families (Monte *et al*., 2004).

Further, complementary feeding should be: **timely**- starting at 6 months of age; **adequate** - foods should be given in right amounts, frequency, consistency and using a variety of foods to cover the nutritional needs of the growing child while maintaining breastfeeding and/or infant formula feeding. **Safety**- foods should be prepared hygienically to minimize the risk of contamination with pathogens. Practice **responsive feeding-** applying the principles of psychosocial care. Feed infants directly and assist them while feeding by themselves. Feed slowly and patiently, and encourage children to eat, but do not force them. If children refuse many foods, experiment with different food combinations, tastes, textures and methods of encouragement. Minimize distractions during meals if the child loses interest easily. Remember that feeding times are periods of learning and love- talk to children during feeding, with eye-to-eye contact (WHO, 2009).

However, during this transition period, introducing the child to CFs, the prevalence of undernutrition increases substantially in many countries including Ethiopia, because of an increase in infections and poor feeding practices (CSA, 2012). This is may be the CFs made from locally available ingredients (locally available cereals and legumes) may have the susceptibility to get contaminated by different contaminants like aflatoxin, *C. sakazakii*, coliforms, *E. coli* and other pathogens that are the major route of transmission of diarrhea among infants and young children. Globally, diarrhea is a major cause of morbidity in infancy (Thurnham, 2013). In Ethiopia, dehydration from diarrhea is a major cause of death in infancy and childhood and 13% of children under age five were reported to have had diarrhea (CSA, 2012).

Therefore, proper maternal practices regarding the management, preparation, administration and storage of CFs may reduce their contamination. All stages and processes in the food chain must be completed according to correct practices, ensuring that hazards are controlled and eliminated or reduced to acceptable levels (Monte *et al*., 2004). Further, mothers/caregivers are provided with appropriate guidance regarding optimal feeding of infants and young children (Baye *et al*., 2013; Dewey & Brown, 2003; Gibson *et al*., 2009; WHO, 2005).

2.2 Food safety knowledge and practices

Foods are generally considered safe, provided that care is taken during primary production, processing, storage, handling and preparation. Food safety involves all aspects from farm- to-table. Therefore, food safety is defined as an assurance that food will not cause harm or sickness to the consumer when it is prepared and/or eaten according to its intended use (FAO/WHO, 2009). Each year, millions of people worldwide suffer from food-borne diseases and illnesses resulting from the consumption of contaminated food, which has become one of the most widespread public health problems in the world (WHO, 2000).

In developing countries, many people are poisoned because of the consumption of foods produced under poor hygienic conditions: lack of hygiene education, inappropriate food storage conditions, lack of cleaning, aflatoxin contamination, microbial contamination, pesticide residue and others. The presentation of food not stored under hygienic conditions, the demand for cheap food and failure to provide the required care while preparing food results in food poisoning in developed countries as well (Medeiros *et al*., 2004). As a result, foodborne diseases are responsible for considerable morbidity and mortality globally (Linscott, 2011). Therefore, safety of food continues to be of paramount importance for not only consumers but also the entire food industry and regulatory authorities.

Occurrence of foodborne illnesses is at times attributed to the improper handling of the food items at consumers' homes (Redmond & Griffith, 2003). Mishandling of food can occur during preparation, handling and/or storage of food and numerous studies have shown that the mishandling of food occurs because consumers have inadequate knowledge about food handling practices (Redmond & Griffith, 2003). Hence, educating consumers and food handlers on safe food handling practices can achieve prevention and control of foodborne illnesses (Jevsnik *et al*., 2008).

Even though a lack of knowledge on the part of food handlers has contributed to the prevalence of food-borne diseases (WHO, 2000), and training and education are essential in supplying this knowledge, it does not automatically translate to safe food handling practices (Clayton & Griffith, 2008). Research is conducted to ascertain the level of knowledge of food handlers concerning safe food handling practices and the actual practices that take place in the work environment so that relevant and effective food training programs can be planned. Some scholars have addressed only one variable (knowledge or practice), while others have combined knowledge, attitude, and practices as variables of interest.

Many studies have been conducted in different countries to assess food handlers' food safety knowledge on areas such as hand washing, temperature control, cross contamination, food storage, and some aspects of food microbiology. Hislop & Shaw (2009) conducted a study in Edmonton, Canada to determine the food safety knowledge of food handlers in the food service industry. Knowledge was assessed by using standardized, self-administered questionnaire distributed by environmental health officers during site inspection.

Studies have also assessed the knowledge and practices of the people on issues related to mold and aflatoxin contamination in various types of foodstuffs in many countries across the world. In Malawi, a study by Matumba *et al*. (2016) who reported that the people especially females do not have the knowledge about the health implications associated with moldy food and feedstuffs. Another study by Sanders *et al*. (2015) reported that the Flemish population lacked awareness about the toxic effects of mycotoxins. Mboya and Kolanisi (2014) also reported that people in Tanzania used fungal infected staples for food, implying that people are not fully aware of health hazards associated with the ingestion of mycotoxins. A similar study by Ezekiel *et al*. (2013) in Nigeria reported that about 85% of peanut cake consumers did not have awareness about the health impacts of aflatoxin. A study by Awuah *et al*. (2008) also reported that people in Ghana are unaware of the harmful effects of aflatoxins.

Therefore, awareness creation of the people on issues related to mold and aflatoxin contamination of foods in general and CFs in particular is very critical to prevent the health impacts of aflatoxins.

2.3 Microbial safety of CFs

Contaminants are a vast subject area of food safety and quality and can be present in our food chain from raw materials to finished products. Contaminants can be classified as; physical, microbial, and chemical (mycotoxins, acrylamide, heavy metals, pesticides, nitrates/nitrites, polyaromatic hydrocarbons and others) contaminants. Microbial pathogens, which can come from different stages of production and storage, may be present in infant formulas, babay foods and CFs. Contamination of baby food with microbial pathogens can raise important health impacts in a neonate's or an infant's and may lead to severe problems or even death as the immune functions as well as other defense mechanisms of neonates/infants are not as well-functioned as adults (Erkekoglu *et al*., 2009). *C. sakazakii*, Coliforms, *E. coli* and others are the common microbial pathogens that contaminate CFs.

2.3.1 *Cronobacter sakazakii*

Cronobacter species has been one of the well known microbial pathogens which can contaminate dry baby foods and infant formulas and cause severe intoxications and infections (Erkekoglu *et al*., 2009; Chap *et al*., 2009; Yao *et al*., 2016). Cronobacter species are Gram-negative motile, peritrichous non-spore forming, facultative anaerobic bacterium. It is an opportunistic foodborne pathogens of the family Enterobacteriaceae and closely related to Enterobacter and Citrobacter. It consists of *C. sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. turicensis*, *C. dublinensis*, *C. condimenti*, and *C. universalis*. Cronobacter species cause rare but severe infections in patients of all age groups. In adults, Cronobacter species are often associated with nosocomial infections, including pneumonia, septicemia, wound infections, and osteomyelitis, while causing invasive disease in young infants and neonates (Patrick *et al*., 2014). Among the seven identified Cronobacter species, *C. sakazakii* plays a prominent role due to it causing life-threatening infections in neonates (Jason, 2012).

C. sakazakii, formerly *Enterobacter sakazakii*, was one of Cronobacter species and accepted as a new bacterial genus in 2007 (Iversen *et al*., 2007). It is an opportunistic pathogen that can cause infant meningitis, septicaemia, and necrotizing enterocolitis and result in mortality rates of 40–

80% among infected patients (WHO, 2006). The first cases attributed to this organism occurred in 1958 in England (Urmenyi & Franklin, 1961).

C. sakazakii mainly infects neonates, especially premature or with low birth weight individuals, although recent studies have demonstrated that this pathogen can also infect adults, particularly elderly individuals (Bowen & Braden, 2006). *C. sakazakii* inhabits a wide variety of foods including milk, cereals, cheese, dried foods, meats, water, vegetables, bread, tea, herbs, spices, beverages and drinks (Friedemann, 2007; Vojkovska *et al*., 2016; Yao *et al*., 2016). It has been also isolated from a diverse range of enviromnents including soil, rats, flies, milk powder factories, chocolate factories, livestock facilities, and food factories (Gurtler *et al*., 2005; Kandhai *et al*., 2004a; Kandhai *et al*, 2004b). In Ethiopia, *C. sakazakii* was also detected in underground waters used for drinking in Jimma town (Ali *et al*., 2012).

However, previous reports have indicated that milk- based powdered infant formula (PIF) was the primary source and vehicle for *C. sakazakii* outbreaks (CDC, 2002; Li *et al*., 2016), thus, increased attention has been devoted to monitor this pathogen in PIF in many countries (Norberg *et al*., 2012). Further, the US Food and Drug Administration (FDA, 2002) has issued an alert to health care professionals about the risk associated with *C. sakazakii* infections among neonates fed with milk-based PIF. The alert stated that a major contribution to the avoidance of *C. sakazakii* infections in premature babies and neonates is the prevention of contamination of milk-based PIF during production and bottle preparation as the two main routes by which these bacteria can enter infant formula are intrinsic contamination either through contaminated ingredients and/or through external contamination during reconstitution and handling (Mullane *et al*., 2006). This has resulted in increased efforts to implement appropriate strategies to reduce health risks associated with the use of PIF.

In low-income countries, most infants are given cereal-based CFs prepared at the household level. On contrary to PIF, little attention has been paid to CFs contamination by *C. sakazakii* (Shaker *et al*., 2007). In addition, CFs produced at community level in Ethiopia has not been evaluated about the risks of *C. sakazakii* contamination. Therefore, evaluating the presence of *C. sakazakii* in the locally prepared CFs is paramount importance in protecting the health of young children in the study areas. CFs should be free from *C. sakazakii* contamination.

C. sakazkii has resistance to environmental stresses; resistance to dry and osmotic conditions, heat resistance and tolerance to low pH are linked to the spread of *C. sakazakii* in food (Yan *et al*., 2012). Although *C. sakazakii* occur in several matrices, the technical standard protocol for the detection of C. sakazakii is available only for milk-based powdered infant formula (ISO/TS 22964, 2006; Yan *et al*., 2012). Consequently, combinations of standard methods described in ISO/TS 22964 and a new approach using different selective media were employed for the isolation of *C. sakazakii* from food in several studies (Turcovsky *et al*., 2011; Yan *et al*., 2012). Clinically, *C. sakazakii* have been isolated from cerebrospinal fluid, bone marrow, blood, intestinal and respiratory tracts, urine, ear and eye swabs, and skin wounds (Gurtler *et al*., 2005).

2.3.1 Coliforms and *E. coli*

It takes several steps to get food from the farm to the dining table. This step is called the food production chain. Many opportunities exist for food to get contaminated along the chain (Lynch *et al*., 2009). Microorganisms including pathogens can be introduced to the food during primary production (pre-harvest), at harvest and food animals respectively and at postharvest (consisting of food processing, distribution and marketing, storage, preparation and serving). Pathogenic microbes can potentially be found more or less everywhere; from where they directly or indirectly get introduced into food. Some are sustained in human reservoirs and contaminate the food supply via the excreta of infected humans. Many others are sustained in animal reservoirs and contaminate the food supply because they are present in the flesh, milk or eggs in the living animal or because they are in excreta of infected animals that subsequently contaminate the foods we eat. Some persist in the environment or in multiple hosts and can contaminate the foods we eat via pathways that reflect the variety of ecosystems that make up our food supply (Alum *et al*., 2016).

The most common foodborne microbial agents that can cause diarrhoeal diseases and ill-health in humans in general and in young children in particular are *Vibrio cholerae*, *Shigella spp*., *Campylobacter*, *Salmonella spp*., *E. coli*, Coliforms, *Entamoeba histolytica* and others (Motarjemi *et al*., 1993). Globally, diarrhoea is the second leading cause of death for children under the age of five (Black *et al*., 2003) and is a leading cause of growth faltering and malnutrition. Diarrhea is also one of the causes of morbidity and mortality among children in the developing world. Diarrhoea accounted for a median of 21% of all deaths of children aged under 5 years of age in these areas and countries, being responsible for 2.5 million deaths per year (Kosek *et al*., 2003). In Ethiopia, diarrhoea is most common and 13 % of children under age five were reported to have had diarrhoea (CSA, 2012).

Therefore, maintaining the microbial quality is among key attributes to indicate integrity of food products and hygiene of food processing. Further, coliforms and *E. coli* detection has been adopted by world-class food industry to determine microbiological quality of food products. Detection of *E. coli* suggests contaminations from direct or indirect fecal origins of humans and warm-blooded animals. High counts of *E. coli* and coliform in food samples directly imply poor practices in food handling and production operations in the manufacturing chain (Sousa *et al*., 2002). As a result, total coliforms and *E. coli* enumeration are used as a food-quality parameter (Gonzalez *et al*., 2003).

Coliforms (or total coliforms) are aerobic or facultatively anaerobic, Gram negative, non-sporeforming rods capable of fermenting lactose to produce gas and acid within 48 hr at 32-35^0 C. Coliforms are represented by four or five genera of the family Enterobacteriaceae: Citrobacter, Enterobacter, Escherichia, and Klebsiella. Since the relatively new genus Raoultella was formerly a part of Klebsiella, it could represent the fifth coliform genus (Jay *et al*., 2008). The presence of coliforms has long been thought to indicate fecal contamination, however, recent discoveries regarding this diverse group of bacteria indicates that only a fraction are fecal in origin, while the majority are environmental contaminants. For nearly a century, coliforms have been used as indicator organisms, first in evaluating water for fecal contamination and later in identifying unsanitary conditions in pasteurized dairy products and other foods (Martin *et al*., 2016). The genera Escherichia diverged around 102 million years ago, the genera can split in to *E. adecarboxylata*, *E. blattae*, *E. fergusonii*, *E. hermanii* and *E. vulneris* ancestors. However, *E. coli* is the most studied epecies of Escherichia (Welch, 2006).

E. coli, originally called “Bacterium coli commune,” was first isolated from the feces of a child in 1885 by the Austrian pediatrician Theodor Escherich. E. coli are Gram-negative, rod-shaped facultatively anaerobic bacteria. They are approximately 0.5 µm in diameter and 1.0–3.0 µm in length. Within the periplasm is a single layer of peptidoglycan. The peptidoglycan has a typical subunit structure where the N-acetylmuramic acid is linked by an amide bond to a peptide consisting of L-alanine, D-glutamic acid, meso-diaminopimelic acid and finally D-alanine (Welch, 2006).

E. coli is a common inhabitant of the gastrointestinal tract of humans and animals. *E. coli* can also be found in soil and water as the result of fecal contamination. Hence, *E. coli* is a member of the faecal coliform group and is a more specific indicator of faecal pollution. Some *E. coli* strains are harmless commensals of the intestinal tract while others are major pathogens of humans and animals. The pathogenic *E. coli* are divided into those strains causing disease inside the intestinal tract and others capable of infection at extra-intestinal sites (Kaper et *al*., 2004).

Pathogenic *E. coli* are capable of causing disease in healthy individuals. Three general clinical syndromes can result from infection with one of these pathotypes: enteric/diarrhoeal disease, urinary tract infections (UTIs) and sepsis/meningitis. The most common intestinal pathogens that cause different types of disease are: *enteropathogenic E. coli* (EPEC), *enterohaemorrhagic E. coli* (EHEC- the most famous member of this virotype is strain O157:H7, which causes bloody diarrhea and no fever), *enterotoxigenic E. coli* (*ETEC*), *enteroaggregative E. coli* (*EAEC*), *enteroinvasive E. coli* (*EIEC*) and diffusely adherent *E. coli* (DAEC) (Nataro & Kaper, 1998).

Therefore, detecting coliforms and *E. coli* from food and water is very important to determine the microbiological quality. The identification criteria used are production of gas from glucose (and other sugars) and fermentation of lactose to acid and gas within 48 hr at 35^0C (coliforms) and 45.5^0C (*E. coli*) (FDA, 2001). Most of classical techniques to determine the presence of coliforms and *E. coli* are dependent on their unique biochemical properties, including the most probable number technique (MPN), Petrifilm ™ *E. coli*/coliform count (EC) and Chromocult ® coliform agar (CCA) (Venkateswaran *et al*., 1996).

Coliforms and *E. coli* have caused food borne infection through consumption of various types of foods, including; fruits, vegetables, milk and milk products, fish and fish products, meat and meat products, CFs, and others (Gonzalez *et al*., 2003; Martin *et al*., 2016). High counts of coliforms and *E. coli* in food samples directly imply poor practices in food handling and production operations in the manufacturing chain (Sousa *et al*., 2002). Further, consumption of contaminated CFs with coliforms and *E. coli* appeared to be associated with a higher frequency of diarrhoea and malnutrition in children (Kung'u *et al*., 2009; Islam *et al*., 2012). Therefore, the reliable safe supply of CFs that is free from microbial pathogens is important for the health of the young children, economic development, social stability and the government and countries 'image (Alum *et al*., 2016).

As a result, evaluating the safety of the locally prepared CFs and their ingredeints in terms of coliforms and *E. coli* are paramount importance to safeguard the health of young children in the study areas and to provide evidence for safety interventions.

2.4 Mycotoxins

According to an FAO report, around a third of all foodstuffs produced for the world's population are lost from field to consumer, amounting to roughly 1.3 billion metric tons per annum (FAO, 2011). Mycotoxins are one of the most significant contributors to food and feed losses in developing countries, especially in Sub-Saharan Africa (SSA), and became a recurring food safety challenge (Udomkun *et al*., 2017b). As a result, to date, serious concerns are being raised by consumers as well as by health professionals for the presence of various toxigenic fungi or their secondary metabolites (as mycotoxins) in food and feed (Bhat & Reddy, 2017).

Filamentous ascomycetes of genera *Aspergillus*, *Penicillium*, and *Fusarium* are most common types of fungi that are responsible for mycotoxin contamination in different types of foodstuffs (Pereira *et al*., 2014). Contamination of foods by fungi and mycotoxins results in dry matter, quality, and nutritional losses and represents a significant hazard to the food chain (Magan & Aldred, 2007).

Mycotoxins are toxic secondary metabolites produced by the different species of fungi as a by-product of their metabolism (Siegel & Babuscio, 2011). Hundreds of mycotoxins have been identified that bring food safety challenges to the farm-to-table food continuum, but the most frequently occurring and toxicologically recognized classes are aflatoxins (AFs), ochratoxins (OTs), fumonisins (FMs), zearalenone (ZN), trichothecenes (TRC), patulin (PAT), and their derivatives. But in Africa, AFs, OTs, and FMs are considered to be widespread in major dietary and export oriented crops (Chala *et al*., 2014; Mutiga *et al*., 2015; Vismer, *et al*., 2015).

Mycotoxins are generally heat stable and are not destroyed during most normal cooking processes (Bullerman & Bianchini, 2007; Freire & Rocha, 2016). As a result, foods contaminated with mycotoxins are observed as toxic to animals and humans. Mycotoxicosis is the term used for poisoning associated with exposures to mycotoxins. The symptoms of mycotoxicosis depend on the type of mycotoxin, the concentration and duration of exposure, as well as age, health, and sex of the exposed individual (Bennett & Klich, 2003).

Like all toxicological syndromes, mycotoxicosis can be categorized as acute or chronic. Acute toxicity generally has a rapid onset and an obvious toxic response, while chronic toxicity is characterized by low-dose exposure over a long time-period, resulting in cancers and other generally irreversible effects (Roberts *et al*., 2014). Although the main human and veterinary health burdens of mycotoxin exposure are related to chronic exposure (such as; cancer, kidney damage, immune suppression, mutation, and many others) (Sherif *et al*., 2009). In the body, mycotoxins disrupt metabolism of proteins, carbohydrates, and lipids as well as alter growth factor expression and impair growth in children (Gong *et al*., 2003; Gong *et al*., 2004; Gong *et al*., 2008; Khlangwiset *et al*., 2011; Turner *et al*., 2007).

Further, mycotoxin contamination lowers product quality and reduces export values, which may lead to significant economic losses for producing countries. They can also indirectly waste natural resources as well as lead to hunger and malnutrition (Leslie *et al*., 2008). However, mycotoxins have been proven under laboratory experiments not to be necessary for the fungi ‘growth and have been thought to aid in competition against other organisms in habitat (Shwab & Keller, 2008).

Mycotoxin production is mainly dependent on conditions related to climate, crop production, handling, and storage. Unfavorable postharvest and storage conditions are conducive for the perforation of mold and mycotoxin contamination (Paterson & Lima, 2010). Pre-harvest factors such as genotype, water stress, soil conditions, and insect activity have all been found to be influential for the risks of mycotoxin contamination (Wagacha & Muthomi, 2008). Further, socio-economic factors such as unavailability of materials, tools, and equipments as well as poor marketing and transportation systems, lack of information, and inadequate governmental policy, regulations, and legislations can also contribute to the risks of mycotoxin contamination (Udomkun *et al*., 2017b).

The control of mold growth and following mycotoxin production in food crops requires a variety of risk management options. These range from prevention of mold growth through good agricultural practices (GAP), setting of regulatory limits in grains destined for food use, a combination of surveillance, regulatory and quality assurance procedures. In addition, Hazard Analysis of Critical Control Point (HACCP) system, together with good manufacturing practices (GMP) from farm-to-table should be implemented (Magan & Olsen, 2004; Waliyar *et al*., 2008).

Mycotoxin-producing fungi are ubiquitous and cannot be easily eliminated, but contamination can be mitigated to acceptable levels for consumption through strategic interventions. Several pre- and postharvest strategies have been developed to prevent the risks of mycotoxin contamination in food. At the pre-harvest level, these interventions focus on the reduction of fungal infection in the field, through GAPs (Cleveland *et al*., 2003), host plant resistance (Brown *et al*., 2013) and bio-control (Tran-Dinh *et al*., 2014). Postharvest interventions include GMPs focusing on timely harvest and prompt drying as well as transportation and storage improvements (Turner *et al*., 2005). Further, implementing HACCP system, together with GMP is important in preventing the risks of mold and mycotoxin contamination in food from farm-to-table (Magan & Olsen, 2004; Udomkun *et al*., 2017b).

2.4.1 Aflatoxins

Aflatoxins are clearly the most widely studied of all mycotoxins. Knowledge of their existence dates from 1960, when more than 100,000 turkey poults died ("turkey X disease") in England after eating peanut meal imported from Africa and South America. From the poisonous feed were isolated *Aspergillus flavus*, and a toxin produced by this organism that was designated aflatoxin (Aspergillus flavustoxin; A-fla-toxin) (De Iongh *et al*., 1962). Aflatoxins are considered as the most important group of mycotoxins in the world food supply. They are a group of chemically related toxins produced primarily by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. In nature, Aspergillus flavus is one of the most abundant and widely distributed soil-borne molds found on earth (Yu & Keller, 2005). *Aspergillus flavus* produces asexual spores, conidia and the asexual fruiting body, sclerotia, which is a resistant structure that enables the strain to survive under harsh conditions (Amaike & Keller, 2011).

Aflatoxins contaminate multiple staple foods, including cereals (such as maize, sorghum, wheat, barley), groundnuts, cotton seed, tree nuts, cheese, spices, and a long list of other food and feed commodities (Ayalew *et al*., 2006; Bennet & Klich, 2003; Chala *et al*., 2014; Pitt & Hocking, 2009). Aflatoxin production is favored particularly by warm climates. Exposure of the mature crop to favourable temperature and moisture conditions, either in the field or during transportation and storage, could be associated with increased toxin production. The optimum temperatures for aflatoxin production (24-30°C) can differ among *Aspergillus flavus* and/or *Aspergillus parasiticus* isolates while optimum temperature for growth ranges from 30-35°C (Lahouar *et al*., 2016; Mousa *et al*., 2013).

Aflatoxin is most prevalent in crops in tropical and subtropical regions of the world, and may occur in the field and post-harvest under suboptimal storage conditions. It has been estimated that 5 billion people worldwide are exposed to uncontrolled aflatoxin in the diet (Strosnider *et al*.,2006). Developing nations, including most of Africa, Latin and South Americas, and Asia are identified as high-risk areas for aflatoxin exposure, leading to aflatoxicosis (Williams *et al*., 2004).

There are six predominant aflatoxins, named aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), aflatoxin M1 (AFM1), and aflatoxin M2 (AFM2) (Chin *et al.*, 2012; Garrido *et al.*, 2003). AFB1, AFG1, AFB2, and AFG2 (in order of decreasing toxicity) are the four major naturally occurring aflatoxins found in food. While AFM1 and AFM2 are oxidative metabolized products of aflatoxins B1 and B2 and usually excreted in milk (both animal and human), urine and faeces (Adejumo *et al.*, 2014; Gezachew *et al.*, 2016; Mykkanen *et al.*, 2005).

In the above nomenclature, 'B' and 'G' are used to denote compounds that fluoresce blue or green, respectively, under ultraviolet light. The 'M1' and 'M2' compounds are not found in cereals and cereal based products, but are metabolites expressed in milk of mammals whose diet was contaminated by aflatoxins B1and B2, respectively. Finally, the '2' numbered aflatoxins are structural isomers missing one double bond, as compared to the respective '1' numbered molecule, showed in Figure 2.1 (Klich, 2007). Aspergillus flavus normally produces aflatoxin Bs, while Aspergillus parasiticus produces both aflatoxin Bs and Gs. Other important species that produce both aflatoxins B and G include *Aspergillus toxicarius*, *Aspergillus nomius*, *Aspergillus bombycis*, *Aspergillus parvisclerotigenus*, *Aspergillus minisclerotigenes*, and *Aspergillus arachidicola* (Klich, 2007; Varga *et al.*, 2009).

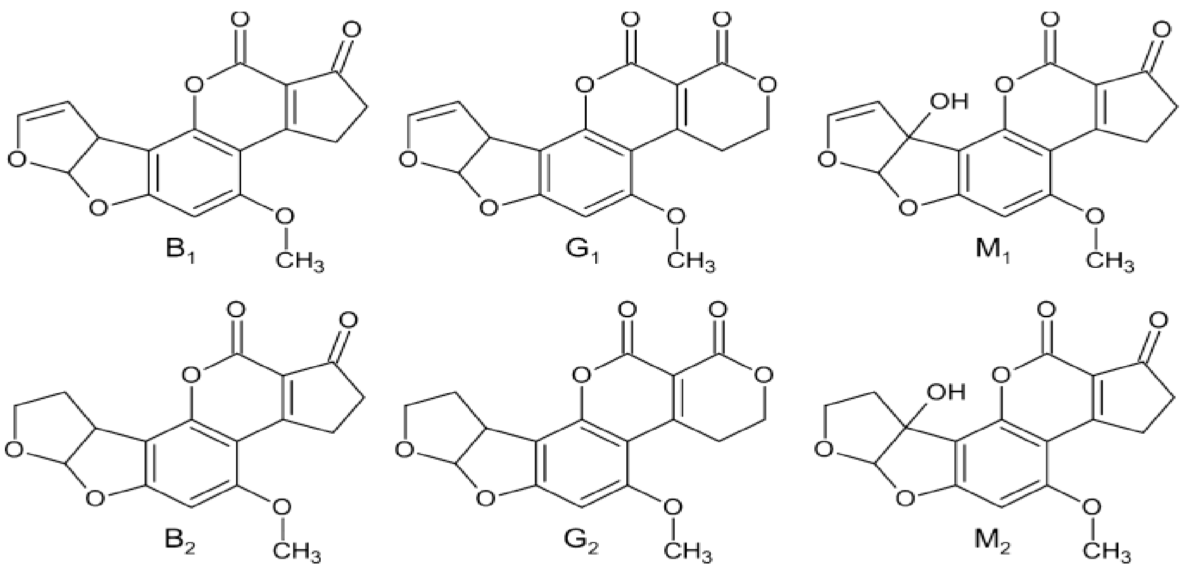

Figure 2.1 The chemical structures of AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2.

Different generalized occurrence ratios of the four aflatoxins (AFB1:AFB2: AFG1:AFG2- 1.0:0.1:0.3:0.03) have been reported (Kensler *et al.*, 2011). However, most agree that AFB1 concentration generally exceeds half of the sum of the aflatoxins and that AFB2 and AFG2 occur in the lowest concentrations. In the same regard, several countries have set separate regulatory limits for AFB1 at half the regulatory limit of the sum of the four aflatoxins (Van Egmond & Jonker, 2004). Likewise, analytical methods for quantifying AFB1 alone in various matrices have been developed (Yu *et al*., 2013). The ratio of aflatoxin B and G concentrations is greatly influenced by temperature and water activity (Schmidt-Heydt *et al*., 2010) and population ratios of fungal strains on given matrices (Wilson & King, 1995). Differences in amount produced of aflatoxin B1 or G1 under various environmental conditions is obviously attributable to the adaptation to changing environments.

However, a study by Matumba *et al*. (2015a) reported that the ratio of AFB1 to AFG1(1.09:1.0) is different from the previous reports (1.0:0.3), which is an indication of considering total aflatoxins rather than AFB1 alone for legislation purposes. That is why in our study total aflatoxins (sum of AFB1, AFB2, AFG1, and AFG2) were determined in the CFs and their ingredients instead of AFB1.

2.4.1.1 Health impacts of aflatoxins

The main route of exposure to aflatoxin is through the direct consumption of contaminated food. Aflatoxin exposure can occur throughout the life course, beginning in utero through transplacental exposure (Hernandez-Vargas *et al*., 2015). Breast milk is a pathway of exposure for young children at breastfeeding (Magoha *et al*., 2014); even though, the AFM1 found in milk is less toxic than AFB1 found in food. In Africa, CFs are often cereal and legume-based, both of which are susceptible to aflatoxin contamination and children's exposure increases during the complementary feeding period (Gong *et al*., 2003).

Aflatoxicosis is the poisoning that results from ingesting aflatoxins. Two forms of aflatoxicosis have been identified: acute and chronic. A review of the literature across all species provides clear evidence that the dose and duration of exposure to aflatoxin clearly have a major effect on the toxicology and may cause a range of consequences:1) large doses lead to acute illness and death, usually through liver cirrhosis; 2) chronic sublethal doses have nutritional and immunologic consequences; and 3) all doses have a cumulative effect on the risk of cancer (Williams *et al*., 2004).

High level exposure of aflatoxin that occurs over a relatively short period of time is recognised as causing acute aflatoxicosis. For example, in Eastern Kenya in 2004, 317 individuals were diagnosed with acute liver failure of which 125 (39%) subsequently died as a result of acute aflatoxicosis (Azziz-Baumgartner *et al*., 2005). The level of aflatoxin exposure (AF-alb adduct) was higher in patients than in healthy individuals. A case-control study showed that the outbreak in Kenya may have been triggered by consuming aflatoxin contaminated home-grown maize (Azziz-Baumgartner *et al*., 2005).

The symptoms of acute severe aflatoxicosis include hemorrhagic necrosis of the liver, bile duct proliferation, edema, and lethargy. Animal studies have found two orders of magnitude difference in the median lethal dose for AFB1. Susceptible species such as rabbits and ducks have a low (0.3 mg/kg) median lethal dose, whereas chickens (18 mg/kg) and rats have greater tolerance. Adult humans usually have a high tolerance of aflatoxin, and, in the reported acute poisonings, it is usually the children who die (Eaton & Groopman, 2013).

Chronic aflatoxicosis due to low dose aflatoxin exposure over a long period of time, is more prevalent than acute aflatoxicosis. The most well-established health effect of chronic exposure is hepatocellular carcinoma (HCC). Other chronic toxic health effects include growth faltering in children (Gong *et al*., 2002; Gong *et al*., 2004; Turner *et al*., 2012) and immune suppression (Jiang *et al*., 2005; Turner *et al*., 2003; Turner *et al*., 2012).

i. Hepatocellular carcinoma

HCC is the most prevalent primary malignant tumor of the liver and is accepted as one of the major malignant diseases in the world today (Hamid *et al*., 2013). HCC, the commun type of liver cancer is much more common in men than in women. In men, it is the second leading cause of cancer death worldwide and in less developed countries. In more developed countries, it is the sixth leading cause of cancer death among men. An estimated 782,500 new liver cancer cases and 745,500 deaths occurred worldwide during 2012, with China alone accounting for about 50% of the total number of cases and deaths. The highest incidence rates are observed in Asian and African countries (Torre *et al*., 2015).

Dietary exposure to aflatoxins is among the major HCC risk factors. Aflatoxin B1, which is a genotoxic hepatocarcinogen, which presumptively causes cancer by inducing DNA adducts leading to genetic changes in target liver cells. AFB1 is metabolized by cytochrome-P450 enzymes to the reactive intermediate AFB1-8, 9-epoxide (AFBO) (Figure 2.2) which binds to liver cell DNA, resulting in DNA adducts. DNA adducts interact with the guanine bases of liver cell DNA and cause a mutational effect in the P53 tumor suppressor gene at the codon 249 hotspots in exon 7, which may lead to HCC (Hamid *et al*., 2013) (Figure 2.3). In addition to AFB1, Hepatitis B virus (HBV) and hepatitis C virus (HCV) are also possible risk factors for HCC. Especially HBV is highly prevalent in Africa and South Asia, and can synergistically interact with AFB1, resulting in an increased risk of HCC (Wu *et al*., 2009).

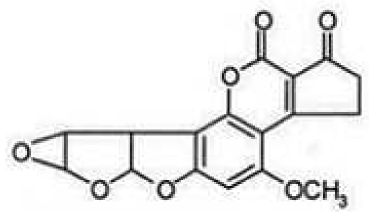

Figure 2.2 The structure of AFB1-8, 9- epoxide.

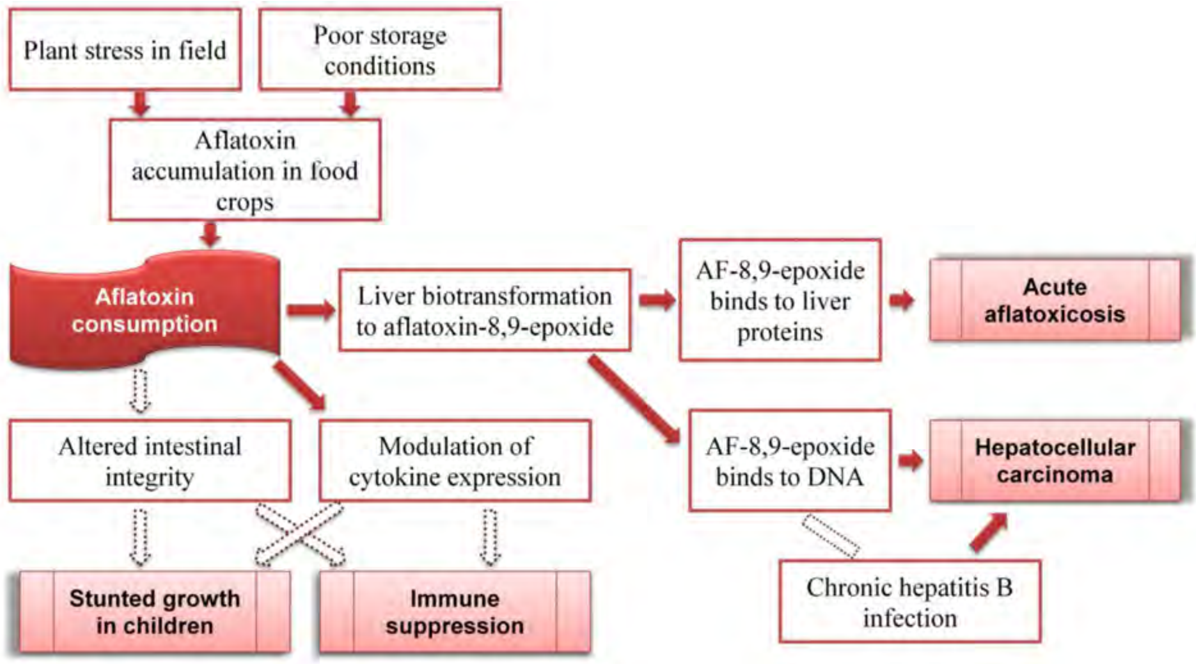

Figure 2.3 Aflatoxin disease pathways in humans (Source: Wu, 2010).

A systematic review and meta-analysis of epidemiological studies carried out in sub-Saharan Africa, China and Taiwan, examined the population attributable risk (PAR) of aflatoxin-related HCC. PAR represents the number or proportion of patients in a population that would not occur if the risk factor were removed. It was found that the PAR of aflatoxin-related HCC was 17%, but was higher in HBV positive populations (21%) in comparison with HBV negative populations (8.8%). This indicates that reducing dietary aflatoxin exposure could reduce the risks of HCC in high risk areas (Liu *et al*., 2012). Another study in China by Chen *et al*. (2013) also reported that a reduction in aflatoxin exposure by changing diet (from maize based to a rice based diet-typically lower aflatoxin contamination) together with control of HBV can achieve a large reduction in liver cancer prevalence.

Aflatoxin has also been implicated in the aetiology of other liver diseases including cirrhosis (Kuniholm *et al*., 2008) and hepatomegaly (Gong *et al*., 2012). A study in Kenya by Gong *et al*. (2012) reported that the prevalence of hepatomegaly, a firm form of liver enlargement, increased in children with higher aflatoxin exposure. This is consistent with the fact that the liver is the key target organ for aflatoxin toxicity. Therefore, preventing the CFs from aflatoxin contamination could substantially decrease the burden of chronic liver disease in the young children.

ii. Growth faltering in children

Growth impairment in children is a prevalent public health problem in low- and middle-income countries worldwide, and is associated with a wide variety of factors such as poor nutrition, poor hygiene, socioeconomic status, local political instability, repeated infectious diseases, and environmental toxins (Black *et al*., 2008). Aside from adverse health effects associated with childhood growth impairment, such as cognitive impairment and increased risk of infectious diseases and death, there are also economic consequences (Victora *et al*., 2008).

Childhood growth performance is usually measured by one or more of three indicators: height for age, weight for age, and weight for height. Based on the WHO definitions, children whose heights for ages, weights for ages, and weights for heights are below WHO growth references (z-scores) are considered to be stunted, underweight, and wasted, respectively (WHO, 2006). Wasting is an indicator of deficits in tissue and fat mass, which may be caused by acute malnutrition, where as stunting is regarded as an indicator of chronic malnutrition. Underweight is an indicator of acute or chronic malnutrition or a combination of both.

Stunting is a widely-used indicator of chronic malnutrition in early childhood, including malnutrition during fetal development due to poor maternal nutrition. Therefore, reducing risk factors for stunting in children particularly during the first 1000 days of life could be very critical (Gong *et al*., 2016). Among the risk factors associated with stunting, aflatoxin emerges as playing a potentially important contributory role (Gong *et al*., 2002; Gong *et al*., 2004; Gong *et al*., 2016; IARC, 2016; Khlangwiset *et al*., 2011; Smith *et al*., 2015; Turner *et al*., 2007; Wild *et al*., 2015). The weight of evidence linking aflatoxin with growth impairment has been increasing over the last five decades of research: first primarily in animal studies, and in the last decade increasingly in epidemiological studies.

As is the case with HCC, childhood stunting is prominent in world regions where foodborne aflatoxin exposure is high: South and East Asia, and sub-Saharan Africa. Childhood exposure to aflatoxin can occur in utero, in mothers' breast milk, and particularly in complementary foods (Khlangwiset *et al*., 2011).

Aflatoxin exposure can occur in utero through a transplacental pathway (Turner *et al*., 2007), and that higher exposure levels in utero have been associated with lower birth weights (Abdulrazzaq *et al*., 2004) and stunted child growth (Turner *et al*., 2007). Breast milk is also the the potential source of aflatoxin exposure for young children if the mother is exposed to AFB1 contaminated foods. AFM1, the hydroxylated metabolite of AFB1, is typically detected in breast milk (Adejumo *et al*., 2013; Atasever *et al*., 2014; Iha *et al*., 2014; Jafari *et al*., 2017; Polychronaki *et al*., 2006; Polychronaki *et al*., 2007; Radonic *et al*., 2017). However, only a few epidemiological studies have investigated the relationship between AFM1 in breast milk samples and child growth impairment. A study in Tanzania by Magoha *et al*. (2014) reported that a small but significant ($P<0.05$) inverse association was observed between AFM1exposure levels and height for age Z-score. This study highlights the potential for exposure of AFM1 from breast milk contributing to child growth impairment. However, more research is needed to draw reasonable conclusions. Hence, breast-feeding should, therefore, not be discouraged based on this limited evidence.

Young children especially in developing countries can also be exposed to aflatoxin during the time when CFs are typically introduced. Since CFs are mostly cereal based which is higly susceptible to the risks of aflatoxin contamination (Egal *et al*., 2005). Further, exposure levels relative to body weight are higher for children than for adults and the rapid growth that occurs, and the additional nutrients required during this time period, reflects critical time for the impact of aflatoxin on growth. As a result, aflatoxin exposure during this critical period may be an underlying determinant of impaired child growth. A study in Benin and Togo by Gong *et al*. (2002) reported that aflatoxin exposure levels increased when children started on complementary food and aflatoxin exposure was inversely correlated with HAZ, WAZ and WHZ after adjustment of confounding factors. Another study in Benin, West Africa by Gong *et al*. (2004) also reported that a strong negative correlation ($p< 0.0001$) was observed between AF-alb and height increase over the 8-month follow-up of young children consuming maize based porriage as a major CF.

Therefore, taken together these studies pick out and emphasize that complementary feeding is a critical period for the growth impact of aflatoxin exposure.

Taken together, the studies described above suggest that aflatoxin exposure contributes to child growth impairment independent of, and together with, other risk factors that may cause stunting. Several possible mechanisms have been proposed, certainly, one or more may be relevant to the role of aflatoxins in growth impairment. A study by Gong *et al*. (2008) reported the potential mechanisms for the aflatoxin child growth impairment, that is, the immunosuppressive effect of aflatoxin exposure that may increase infection susceptibility, consequently impairing nutritional status through appetite suppression and reduced nutrient absorption. Smith *et al*. (2012) in their review also postulated that exposure to aflatoxin may promote intestinal damage through protein synthesis inhibition, consequently leading to a reduction in the absorption of essential nutrients and subsequent impaired growth. Another study by Castelino et al. (2015) suggested that aflatoxin exposure may disrupt the insulin-like growth factors (IGF) pathway through liver toxicity. In the study in Kenya, AF-alb concentrations were inversely associated with IGF1 levels (P= 0.039) and IGF binding protein 3 levels (P= 0.046). A path analysis showed that lower IGF1 levels explained about 16% of the effect of aflatoxin on child height. Therefore, an intervention study targeted at aflatoxin reduction should be warranted.

iii. Immune suppression

The immunosuppressive effects of aflatoxin, which include reduced antibody production, increased susceptibility to infectious diseases such as diarrhoea and reduced cell-mediated immunity (CMI) (Gong *et al*., 2016; Mohsenzadeh *et al*., 2016). Further, the immunosuppressive effects of aflatoxin exposure may accelerate the progression of HIV (Jiang *et al*., 2008). There is evidence to show that HIV positive individuals with high aflatoxin exposure, have lower levels of immune markers such as CD4+ T regulatory cells (P= 0.009) and naive CD4 + T cells (P = 0.029), as well as lower percentages of type B lymph cells (P= 0.03), compared to HIV positive individuals with low aflatoxin exposure (Jiang *et al*., 2008). In the same study, negative relationships were observed between AF-alb and perforin-expressing CD8+ T cells (P= 0.045), T regulatory cells (P= 0.002) and B cells (P= 0.012) among HIV positive individuals. However, more studies are needed, in particular with respect to the effect on antibody responses and in non-HIV populations particularly in young children, to determine whether aflatoxin plays a major role in suppressing immune status.

Further, aflatoxin can have serious effects on childhood nutrition. At the levels of exposure in some developing countries, child nutrition and development are interfered with selenium absorption. Animal studies show that aflatoxin also interferes with vitamins A and D, iron, selenium, and zinc nutrition (Costanzo *et al*., 2015; Watson *et al*., 2016; Williams *et al*., 2004). Therefore, addressing the issues around aflatoxin contamination may be critical to improving world health.

2.4.2 Aflatoxin biomarkers

For the past decades, two main methods are used to assess human exposure to aflatoxins in the context of epidemiological studies. These are the use of questionnaires estimating quantity and frequency of intake of dietary foods suspected to be contaminated with aflatoxins or laboratory analysis of aflatoxins in representative food samples collected from the target areas, or both (Qian *et al*., 1994).

These approaches have some limitations, which may lead to misclassification of individual exposure, a major source of error in environmental epidemiological studies. First, aflatoxin contamination in food and food commodities is heterogeneous with marked seasonal variations and thus, estimation of aflatoxin levels through questionnaires or laboratory analysis is extremely difficult (Turner *et al*., 2012; Williams *et al*., 2004). Second, aflatoxin exposure leading to disease outcome might have happened years earlier making it difficult to link the current food contamination levels to disease (Lui & Wu, 2010). Third, humans can be exposed to aflatoxins through other routes like physical contact via the skin and inhalation (Boonen *et al*., 2012). Finally, there are individual variations in terms of absorption, distribution, metabolism and excretion of aflatoxins (Arcella & Leclercq, 2004).

In order to circumvent the above-mentioned limitations, biomarkers have been proposed as a suitable alternative (Turner *et al*., 2012). Biomarkers are typically used as indicators of exposure, effect or susceptibility. A biomarker of exposure refers to measurement of the specific agent of interest, its metabolites, or its specific interactive products in a body compartment or fluid, which indicates the presence and magnitude of current and past exposure. A biomarker of effect indicates the presence and magnitude of a biological response to exposure to an environmental agent. Such a biomarker may be an endogenous component, a measure of the functional capacity of the system, or an altered state recognized as impairment or disease. A biomarker of susceptibility is an indicator of a measure of an inherent or acquired ability of an organism to respond to the challenge of exposure. Measures of these biomarkers have great utility in addressing the relationships between exposure to environmental toxins and development of clinical diseases and in identifying those individuals at high risk for the disease. In this context, aflatoxin biomarkers of internal and biologically effective doses have been integral to establishing the etiologic role of this toxin in human HCC and other toxicologic outcomes (Kensler *et al*., 2011).

Biomarkers of exposure to aflatoxin were essential in epidemiological studies that examined the relation between aflatoxin exposure and risk of disease (Kensler *et al*., 2011; Turner *et al*., 2012). Such investigations have determined levels of the serum albumin AFB1-lysin adduct in human blood whilst others have analysed AFB1-N7-guanine (released from AFB1- DNA adduct) or the AFM1 metabolite levels in urine. As the concentration of all these analytes are strongly correlated with aflatoxin intake in chronically exposed individuals they can serve as valid exposure biomarkers (Turner, 2013). Thus, availability of human specimen (blood or urine) and analytical resources will affect the choice of a suitable biomarker. Blood sampling is invasive and requires medical personal, whilst non-invasive sampling of body fluids (e.g. urine or breast milk) is easier to perform in field studies and often better accepted by the participants (Figure 2.4). Thus, in general urine is the most widely chosen matrix for biomarker analysis.

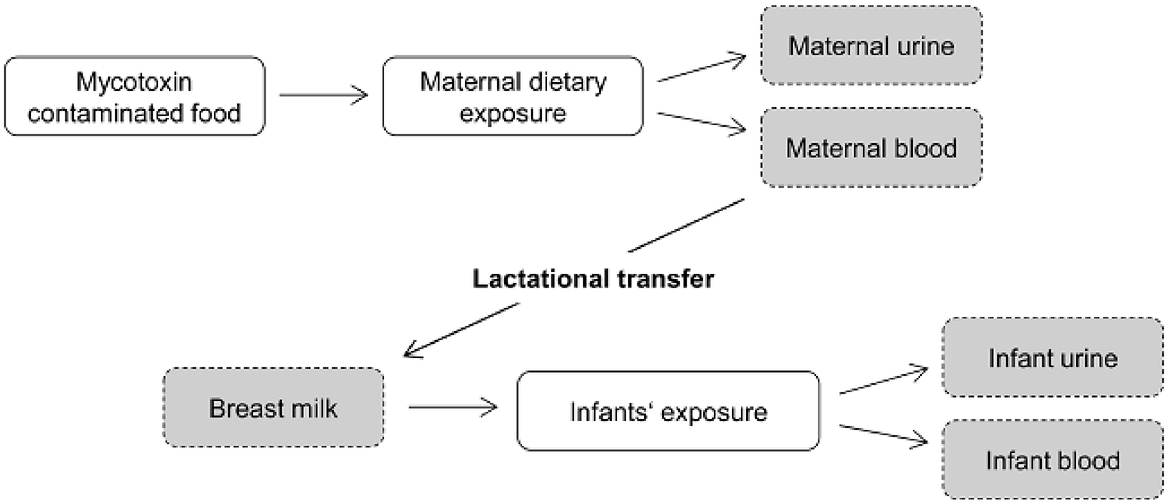

Figure 2.4 Scheme illustrating maternal and infants'exposure toward mycotoxin contaminated food. Potential sampling points to obtain biological fluids for biomonitoring purpose are indicated by dotted boxes. (Source: Warth *et al*., 2016).

Aflatoxin metabolism (Figure 2.5) gives rise to a variety of metabolites (IARC, 1993) including aflatoxin M1, a frequent metabolite in breast milk following maternal exposure to dietary AFB1(Adejumo *et al*., 2013; Atasever *et al*., 2014; Iha *et al*., 2014; Chen *et al*., 2017; Polychronaki *et al*., 2006; Polychronaki *et al*., 2007) and in bovine milk (Gizachew *et al*., 2016). AFM1 is also excreted in urine samples when people are exposed to AFB1 contaminated diets (Abia *et al*., 2013; Ali *et al*., 2015; Ediage *et al*., 2013; Ezekiel *et al*., 2014; Mykkanen *et al*., 2005; Piekkola *et al*., 2012).

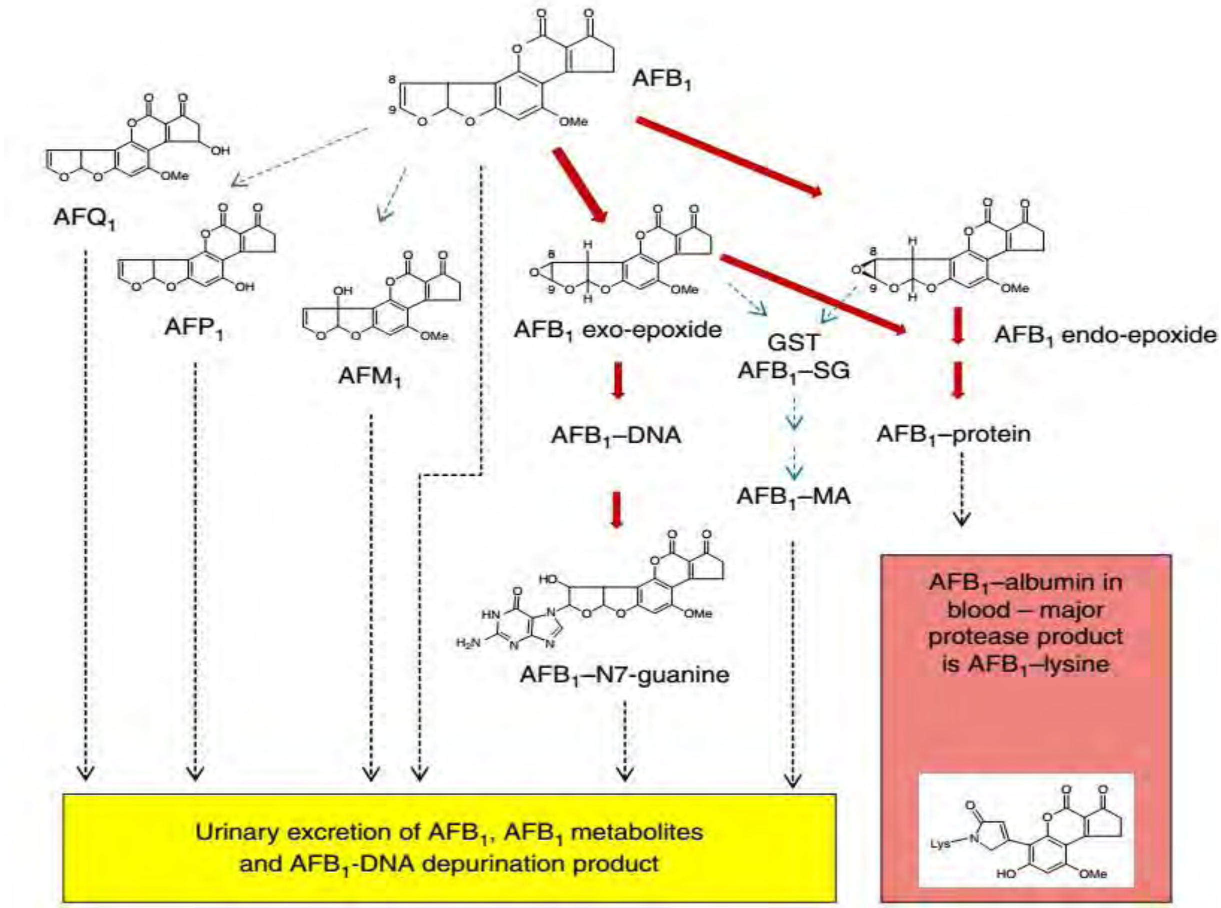

Figure 2.5 AFB1 metabolism and biomarkers. Me, methyl; GST, glutathioneS-transferase; SG, glutathione; MA, mercapturic acid. (Source: Wild & Turner, 2002).

AFM1 has been well established as a biomarker of exposure for the recent ingestion of AFB1 and the most frequently detected urinary aflatoxin (Abia *et al*., 2013; Ali *et al*., 2015; Ediage *et al*., 2013; Ezekiel *et al*., 2014; Mykkanen *et al*., 2005; Piekkola *et al*., 2012). The parent toxins, AFB1, AFB2, AFG1, and AFG2 were also detected in urine samples (Hatem *et al*., 2005; Polychronaki *et al*., 2008).

Analysis of biomarkers in human body fluids covers aflatoxin intake from all dietary sources and exposure by various routes (Fromme *et al*., 2016). However, aflatoxins are genotoxic carcinogens, there is no safe threshold for exposure (Williams *et al*., 2004). Therefore, it is very important to prevent the food from the riks of aflatoxin contamination to ensure that the consumers are free from aflatoxin exposure.

2.4.3 Economic impacts of aflatoxins

In addition to the health impacts, aflatoxins can have economic impacts. Globally, the economic impacts of aflatoxins are large. It can be observed both in terms of the market related losses through selling aflatoxin contaminated commodities, and the human health losses due to adverse impacts of dietary aflatoxin exposure. The economic considerations also include the cost of regulatory and research programs intended to reduce health risks for humans and livestock (Wu, 2015). In the USA alone, annual economic losses of aflatoxin range in the hundreds of millions USD (Wu, 2015). A study by Coulibaly *et al*. (2008) also reported that annual economic losses for US farmers resulting from aflatoxins exceeded 100 million USD on average.

In other parts of the world, both trade-related losses and human and animal health losses are huge (Wu, 2015). In Africa, aflatoxin contamination of crops causes annual losses of more than 750 million USD (Cadwell *et al*., 2001). In Sub-Saharan Africa (SSA), much of the previous studies have focused on trade losses. Only fewer studies have been carried out to determine the additional cost of human effects by aflatoxin contamination such as medical expenses primarily from those suffering from liver cancer as well as indirect costs (pain and suffering, anxiety, and reduction in quality of life) associated with the incidence of aflatoxicosis. A study by Morhason-Bellow *et al*. (2013) reported the problems with quantifying the impact of aflatoxin exposure in SSA to; misdiagnosis, limited access to health care, technical restrictions of medical personnel, and poor infrastructure as well as the low technological level of data systems. However, in Ethiopia, there is no study so far that estimates the economic impacts of aflatoxins.

2.4.4 Management of aflatoxins

Food safety is a critical measure for food security in developing countries, where aflatoxin contamination in key staple crops causes significant postharvest losses, negative impacts on health, and economic welfare as well as direct loss of human life due to fatalities (Lewis *et al*., 2005; Mutegi *et al*., 2013). Aflatoxin contamination can directly reduce availability of food. Farmers who produce contaminated crops may also experience income reduction due to product rejection, lower market value, or exclusion from high-value markets, which translates into reduced access to food. Aflatoxins can also diminish utilization of the contaminated product by either complete market rejection or forced alternate uses. Overall, foods contaminated with aflatoxins also present a clear food security threat regarding their impact on human health, especially concerning the established causal association with liver cancer, synergistic effects with hepatitis B, and potential association with growth inhibition and immune system suppression (Udomkun *et al*., 2017 b).

As a result, different countries and some international organizations have established strict regulations in order to control aflatoxin contamination in food and feeds and also to proscribe trade of contaminated products. The regulations on “acceptable health risk” usually depend on a country’s level of economic development, extent of consumption of high-risk crops, and the susceptibility to contamination of crops to be regulated. Indeed, the established safe limit of aflatoxins for human consumption ranges 4-30 mg/kg. The EU has set the strictest standards, which establishes that any product for direct human consumption cannot be marketed with a concentration of AFB1 and total aflatoxins greater than 2 mg/kg and 4 mg/kg, respectively (EC, 2010).

Similarly, US regulations have specified the maximum acceptable limit for aflatoxins at 20 mg/kg (Wu, 2006). However, if the EU aflatoxin standard is adopted worldwide, lower income countries such as those in Asia and Sub-Saharan Africa will face both economic losses and additional costs related to meeting those standards. This situation requires alternative technologies at pre- and post-harvest levels intended to minimize contamination of food commodities, at least to guarantee that aflatoxin levels remain below safe limits (Prietto *et al*., 2015).

Implementation of innovative technologies is invaluable to address the challenges related to aflatoxins and their effects. Reduction of aflatoxin contamination through knowledge of pre- and post-harvest managements is one of the first steps towards an appropriate strategy to improve agricultural productivity in a sustainable way. This has direct positive effects on enhancing the quality and nutritional value of foods, conserving natural resources, as well as advancing local and international trade by increasing competitiveness. Thefore it is important to identify and document available technologies that can effectively control and minimize aflatoxin contamination along the food chain (Udomkun *et al*., 2017 a).

Different strategies both at pre-harvest and post-harvest stages have been applied to reduce the entry of aflatoxins to the food and feed chains. To date, no single strategy is enough to solve this problem. An integrate management from the field until food or feed processing is necessary to reduce the impact of aflatoxins (Torres *et al*., 2014). Good agricultutural practices (GAP), biological control, sorting technology, treatments with electromagnetic radiation, ozone fumigation, chemical control agents, good manufacturing practices (GMP), implementation of HACCP, and others are some of the common methods used to reduce and/or prevent the risks of aflatoxin contamination (Nguyen *et al*., 2017; Udomkun *et al*., 2017 a; Udomkun *et al*., 2017 b).

2.4.4.1 Biological control

Non-aflatoxin forming strains of *Aspergillus flavus* have been used as a biological control for long-term crop protection against aflatoxin contamination under field conditions. Biocontrol of aflatoxin producing aspergillus strains in the field relies on competitive exclusion, whereby large quantities of non-toxigenic inoculum (*Aspergillus flavus* and *Aspergillus parasiticus*) are introduced into the soil around growing crops and these then compete with toxigenic strains for infection sites on the developing crop (Wild *et al*., 2015). Further, soil inoculation with non-toxigenic strains has a carryover effect, which protects crops from contamination during storage (Atehnkeng *et al*., 2014). The ability of fungus to compete with closely related strains depends on several factors such as pH and soil type as well as the availability of nitrogen, carbon, water, and minerals (Ehrlich, 2014).

The International Institute of Tropical Agriculture (IITA) and the United States Department of Agriculture - Agriculture Research Service together with other partners have been researched in Africa on non-toxigenic biocontrol fungi that act through competitive exclusion strategy (Bandyopadhyay & Cotty, 2013; Grace *et al*., 2015). They have successfully developed several country-specific indigenous aflatoxin biocontrol products generically named as Aflasafe™ which can be used on maize and groundnut (Bandyopadhyay & Cotty, 2013).

This product is an ecofriendly innovative biocontrol technology that utilizes native non-toxigenic strains of Aspergillus flavus to naturally out-compete their aflatoxin-producing cousins (Atehnkeng *et al*., 2014). Aflasafe™ has been shown to consistently reduce aflatoxin contamination in maize and groundnut by 80-99% during crop development, postharvest storage, and throughout the value chain in several countries across Africa (Grace *et al*., 2015). Aflasafe™ products have been registered for commercial use in Kenya, Nigeria, Senegal and Gambia, while products are under development in seven other African nations (Bandyopadhyay *et al*., 2016). Each Aflasafe™ product contains four unique atoxigenic strains of Aspergillus flavus widely distributed naturally in the country where it is to be applied (Atehnkeng *et al*., 2014; Bandyopadhyay *et al*., 2016). Therefore, using Aflasafe™ for the prevention of the risks of aflatoxin contamination may have important benefits in Ethiopia.

2.4.4.2 Good agricultural practices

Aflatoxin contamination may occur in the field before harvest, during harvesting, or during storage and processing. Thus, GAPs both pre-harvset and post harvest strategies are essential elements in the agricultural industry in order to guarantee and maintain the lowest aflatoxin levels possible in cultivated food crops (Alberts *et al*., 2017; Torres *et al*., 2014).

Pre-harvest strategies such as implementation of a crop rotation schedule, adequate preparation of the seed bed for each new crop by ploughing under or by removing old crop debris that may potentially serve as substrate for the growth of aflatoxin producing fungi. Utilizing soil tests is important, if there is need to apply fertilizer and/or soil conditioners to assure adequate soil pH and plant nutrition to avoid plant stress. Subsistence farmers should also apply livestock manure to their fields to enhance stubble breakdown, improve the soil microbiome and aid in plant nutrition (Albets *et al*., 2017; CAC, 2004; Torres *et al*., 2014).

Further, using seed varieties developed for resistance to infecting fungi and insect pests, timely planting to avoid high temperatures and drought stress, avoiding overcrowding of plants by maintaining the recommended row and plant spacing, minimizing insect damage and fungal infection by proper use of registered insecticides, fungicides and other appropriate practices within an integrated pest management programme, controling weeds in the crop mechanically or with the use of registered herbicides or other safe and suitable weed eradication practices, minimizing mechanical damage to plants and fruit during cultivation, ensureing that irrigation is applied evenly and that all plants in the field have an adequate supply of water are also important pre-harvest practices that should be properly implemented to prevent the crop from the risks of mold and aflatoxin contamination (Alberts *et al*., 2017; Torres *et al*., 2014).

Post harvest practices such as harvesting grain at low moisture content and full maturity, ensuring that farm personnel are adequately trained and that all farming equipment is kept clean and functioning properly, to minimize damage to plants and the harvested crop. Containers and vehicles to be used for collecting and transporting the harvested crop from the field to drying and/or storage facilities, should be clean, dry and free of insects, soil and visible fungal growth. Determining crop moisture levels immediately after harvest. Where applicable, dry the crop to the recommended moisture content for storage. Cereals should be dried in such a manner that damage to the grain is minimized and moisture levels are lower than those required to support mould growth during storage (generally less than 12%). Avoid piling or heaping of wet, freshly harvested commodities (commercial; subsistence application). Freshly harvested cereals and nuts should be cleaned or sorted, where possible, to remove damaged kernels/nuts and other foreign matter. Further, storage facilities must be dry, well-vented structures that provide protection from rain, surface or ground water, protection from vermin and birds, and protected from extreme temperature fluctuations (Alberts *et al*., 2017; CAC, 2004; Magan & Aldred, 2007; Torres *et al*., 2014).

2.4.4.3 Sorting technology

Sorting processes seek to eliminate agricultural products with substandard quality. Normally sorting, especially for grains, can be achieved based on differentiation of physical properties such as colour, size, shape, and density as well as visible identification of fungal growth in affected crops. By rejecting damaged and discoloured samples, sorting operations reduce the presence of aflatoxins as well as contaminating materials in food and feed (CAC, 2004; Fandohan *et al*., 2005). Hand-sorting or segregation of crops prior to storage or cooking, is a common practice in many

African countries such as West Africa (Benin), Nigeria, Tanzania and Southern Africa. Sorting, although a tedious task, can significantly remove mycotoxins with an efficiency of 95% or higher in contaminated white maize (Matumba *et al*., 2015).

However, such physical methods are often laborious, inefficient, and impractical for in-line measurements. The application of computer-based image processing techniques is one of the most promising methods for large-scale screening of fungal and toxin contaminations in food and feed. Grains and other agricultural products contain various nutritional substances that are degraded by fungal growth, which in turn influence absorbance spectra of the material. The scattering and absorbance characteristics grains are influenced by the presence of aspergillus flavus in the kernel since fungal development causes the endosperm to become powdery (Pearson *et al*., 2001). Berardo *et al*. (2005) also showed that it was possible to quantify fungal infection and metabolites using Near Infrared Spectroscopy (NIRS). NIRS technique is a fast and non-destructive tool for detecting AFB1 in maize and barley at a level of 20 ppb. Nevertheless, NIRS only produces an average spectrum, which lacks in spatial information from the sample with respect to distribution of the chemical composition (Fernandez-Ibanez *et al*., 2009).

Further, hyperspectral imaging (HSI) is another method that can be employed to monitor both the distribution and composition of mycotoxins in contaminated food samples, especially grains. This method can produce both localized information and a complete NIR spectrum in each pixel (Manley *et al*., 2009). HSI techniques was used to estimate aflatoxin contamination in maize kernels inoculated with Aspergillus flavus spores (Yao *et al*., 2010). Wang *et al*. (2015) also demonstrated the potential HSI based in the Vis/NIR range for quantitative identification and distinction of aflatoxins in inoculated maize kernels.

Another image based sorting technology; a UV light coupled with colour detection system, was successfully detected aflatoxin contaminated figs (Ozluoymak, 2014). This method used the viability of bright greenish-yellow fluorescence (BGYF), which is produced by aspergillus flavus via the oxidative action of peroxidases in living plant tissue (Lundadei *et al*., 2013) as an image screening technique for the classification of aflatoxin contaminated crops.

In addition to sorting; winnowing, shelling, dehulling and milling are important post harvest practices in West and South Africa traditionally practiced by women by hand, using mortar and pestle, grinding rocks and/or wooden sticks to stamp the maize for reducing the risks of aflatoxin contamination. Shelling consists of the removal of the maize kernels from the cob and winnowing is a chaff-removal step to clean the kernels. Dehulling and milling removes parts of the kernel itself, with dehulling the pericarb (outer layers) and during milling the outer layers, hull, pericarp (bran), germ and tip cap are removed to expose the endosperm (Abass *et al*., 2014; Fandohan *et al*., 2006). In a study conducted in Benin, West Africa, the cumulative use of sorting, winnowing, washing crushing and dehulling of maize resulted in a reduction of between 30 and 40% in aflatoxin levels (Fandohan *et al*., 2005). The efficacy of dehulling and milling to reduce aflatoxin levels are related to the higher susceptibility of the outer layers of the maize kernels to fungal perforation and aflatoxin contamination and the physical removal of these (Burger *et al*., 2013).

2.4.4.4 Implementing HACCP system

GAP and GMP are prerequisites and complementary approach to the hazard analysis and critical control point (HACCP) system (Alberts *et al*., 2017). The HACCP system, which is science-based and systematic, identifies specific hazards and measures for their control to ensure the safety of food (CAC, 2009; Hung *et al*., 2015). HACCP is a tool to assess hazards and establish control systems that focus on prevention rather than relying mainly on end-product testing. Any HACCP system is capable of accommodating change, such as advances in equipment design, processing procedures or technological developments (CAC, 2009).

HACCP can be applied throughout the food chain from primary production to final consumption and its implementation should be guided by scientific evidence of risks to human health. As well as enhancing food safety, implementation of HACCP can provide other significant benefits. Further, the application of HACCP systems can aid inspection by regulatory authorities and promote international trade by increasing confidence in food safety. The successful application of HACCP requires the full commitment and involvement of management and the workforce. It also requires a multidisciplinary approach (CAC, 2009).

The HACCP system has been widely adopted by many countries all over the world as well as international organizations, including the World Health Organization and the Food and Agriculture Organization, and is currently a world-recognized preventive management system to maintain food safety (Hung *et al*., 2015). The system comprises seven principles: conduct a hazard analysis, determine the Critical Control Points (CCPs), establish critical limit(s), establish a system to monitor control of the CCP, establish the corrective action to be taken when monitoring indicates that a particular CCP is not under control, establish procedures for verification to confirm that the HACCP system is working effectively, and establish documentation concerning all procedures and records appropriate to these principles and their application (CAC, 2009).

The HACCP program covers the input of the materials, production process, final products, facilities, and personnel at the critical control points (CCPs). However, generally, the HACCP system has two major components: hazard analysis and critical control. Hazard analysis is primarily about systematically assessing the food production process, and identifying the possible hazards "biological", "chemical", and "physical" that may cause the food unsafe. Hazard analysis is conducted at every technical procedure starting from the receipt of materials to the delivery of the final product. The significance of each hazard factor will be determined by the severity of the risk (Hung *et al*., 2015; Lu *et al*., 2014).

Critical control is mainly formulating and managing the controllable points or procedures during the process to minimize the safety hazard of final products based on the results of the hazard analysis (Lu *et al*., 2014). Further, Critical limits must be specified and validated for each CCPs, and care should be taken to ensure that these limits fully apply to the specific operation, product or groups of products under consideration (CAC, 2009).

A study in Ghana by Amoa-Awua *et al*. (2007) reported that the level of total afatoxins in the kenkey samples before the implementation of GMP and HACCP were between 64.1 and 196 µg/kg, while the level sof total aflatoxin were between 17.2 and 14.5µg/kg after implementing GMP and HACCP. Another study by Hung *et al*. (2015) also reported that implementing the HACCP system in food industries can effectively enhance food safety and quality while improving the production management. A similar study by Henry & Xin (2014) also reported that implementing HACCP system ensure the safety of ready-to-use therapeutic foods (RUTFs). Therefore, implementing HACCP in the production of CFs from locally available ingredients in Ethiopia, has paramount importance in minimizing the risks of mold and aflatoxin contamination in the CFs (Annex E).

Further, the application of ISO 22000:2005 in the agri-food chain is becoming important as a food safety management system (FSMS). As a result, African governments could recommend use of ISO 22000:2005 by food-processing sectors and agribusiness to improve their FSMS, which could in turn open up more export opportunities for the African food industry (Kussaga *et al*., 2014).

CHAPTER 3. ASSESSMENT OF MOTHERS' KNOWLEDGE AND PRACTICE TOWARDS AFLATOXIN CONTAMINATION IN COMPLEMENTARY FOODS IN ETHIOPIA: FROM PRE-HARVEST TO HOUSEHOLD

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Assessment of mothers' knowledge and practice towards aflatoxin contamination in complementary foods in Ethiopia: from pre-harvest to household

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RESEARCH ARTICLE

Abstract

This study assessed the knowledge and practices of the mothers in Ethiopia on issues related to aflatoxin in complementary foods (CFs) using structured questionnaires. The study involved 195 mothers from Amhara, Tigray, Oromia, and Southern Nations Nationalities and Peoples (SNNP) regions and addressed a range of issues used to prevent the risks of mould and aflatoxin contamination from farm-to-table. Of the 195 mothers who responded; 186 (95%) were ploughing their land before growing the next crop, 177 (91%) used the crop rotation schedule, 157 (81%) reported the practice of removing old seed heads and stalks used as an inoculum for aflatoxin contamination, 185 (95%) harvested the crops as soon as the crops were matured, 138 (70%) used a threshing method known as 'trampling by hoofed animals'. After threshing, about 124 (64%) respondents had the knowledge and practice of drying cereals and legumes to decrease the moisture content, 134 (68%) used solar drying on a bare ground, 184 (94%) cleaned and disinfected the storage structures before storage. Almost all the respondents practiced the CFs processing steps properly and they used colour, type, odour, insect infestations, mouldiness and all these criteria to select the CFs ingredients. A total of 78 (40%) respondents had the practice of feeding mouldy cereals and legumes to animals, and 89 (46%) of the respondents erroneously believed that roasting can decontaminate the aflatoxin produced from mouldy cereals. As a general conclusion, the majority of the respondents are practicing good agricultural practices in the field, but they use poor storage and processing practices at homes which are susceptible to mould and aflatoxin contamination. Besides, they lack awareness about the toxic effects of aflatoxin on human health. Therefore, awareness creation about the health impacts of aflatoxin and methods of prevention need to be implemented in the study areas.

Keywords: mothers, complementary food, aflatoxin, mould, contamination

1. Introduction

Aflatoxins are toxic secondary metabolites produced by filamentous fungi as a by-product of their metabolism (Calvo *et al.*, 2002). Aflatoxins contaminate around 25% of agricultural products worldwide, with cereals, and groundnuts being the most susceptible (Wild and Gong, 2010; Yard *et al.*, 2013). Contamination of cereals and cereal based products by aflatoxins results in quality and nutritional losses and represents a substantial threat to the food chain (Magan and Aldred, 2007).

Aflatoxins can have both acute and chronic effects to human health when ingested, inhaled, or absorbed through skin (Bennett and Klich, 2003; Reddy *et al.*, 2010). Chronic exposure especially during childhood could be linked to the early onset of hepatocellular carcinoma (Gong *et al.*, 2016; Kirk *et al.*, 2005; Polychronaki *et al.*, 2008; Wild and Gong, 2010; Zain, 2011). Aflatoxin exposure has been also associated with growth faltering (Gong *et al.*, 2002; Gong *et al.*, 2004; Khlangwiset *et al.*, 2011; Turner, 2013, Turner *et al.*, 2007; Wild *et al.*, 2015) and immune suppression in young children (Turner *et al.*, 2003). Aflatoxins impaired growth

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This study assessed the knowledge and practices of the mothers in Ethiopia on issues related to aflatoxin in complementary foods (CFs) using structured questionnaires. The study involved 195 mothers from Amhara, Tigray, Oromia, and Southern Nations Nationalities and Peoples (SNNP) regions and addressed a range of issues used to prevent the risks of mold and aflatoxin contamination from farm-to-table. Of the 195 mothers who responded; 186 (95%) were ploughing their land before growing the next crop, 177 (91%) used the crop rotation schedule, 157 (81%) reported the practice of removing old seed heads and stalks used as an inoculum for aflatoxin contamination, 185 (95%) harvested the crops as soon as the crops were matured, 138 (70%) used a threshing method known as 'trampling by hoofed animals'. After threshing, about 124 (64%) respondents had the knowledge and practice of drying cereals and legumes to decrease the moisture content, 134 (68%) used solar drying on a bare ground, 184 (94%) cleaned and disinfected the storage structures before storage. Almost all the respondents practiced the CFs processing steps properly and they used colour, type, odour, insect infestations, moldiness and all these criteria to select the CFs ingredients. A total of 78 (40%) respondents had the practice of feeding moldy cereals and legumes to animals, and 89 (46%) of the respondents erroneously believed that roasting can decontaminate the aflatoxin produced from moldy cereals. As a general conclusion, the majority of the respondents are practicing good pre-harvest practices, but they use a threshing method (trampling by hoofed animals on a bare floor) and poor storage and processing practices at homes which are susceptible to mold and aflatoxin contamination. Besides, they lack awareness about the toxic effects of aflatoxin on human health. Therefore, awareness creation about the health impacts of aflatoxin and methods of prevention need to be implemented in the study areas.

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3.2 Introduction

Aflatoxins are toxic secondary metabolites produced by filamentous fungi as a by-product of their metabolism (Calvo *et al*., 2002). Aflatoxins contaminate many food crops, but cereals and groundnuts being the most susceptible (Wild & Gong, 2010; Yard *et al*., 2013). Contamination of cereals and cereal based products by aflatoxins results in quality and nutritional losses and represents a substantial threat to the food chain (Magan & Aldred, 2007).

Aflatoxins can have both acute and chronic effects to human health when ingested, inhaled, or absorbed through skin (Bennett & Klich, 2003; Boonen *et al*., 2012; Reddy *et al*., 2010). Chronic exposure especially during child hood could be linked to the early onset of hepatocellular carcinoma (HCC) (Gong *et al*., 2016; Kirk *et al*., 2005; Polychronaki *et al*., 2008; Wild & Gong, 2010; Zain, 2011). Aflatoxin exposure has been also associated with growth faltering (Gong *et al*., 2002; Gong *et al*., 2004; Khlangwiset *et al*., 2011; Turner, 2013, Turner *et al*., 2007; Wild *et al*., 2015) and immune suppression in young children (Turner *et al*., 2003). Aflatoxins impaired growth by targeting the intestinal tract and inducing environmental enteropathy (EE). Reduced nutrient absorption and chronic systemic immune activation are therefore the possible pathways by which EE may cause growth retardation in children (Smith *et al*., 2012). Acute exposure known as aflatoxicosis can lead to vomiting, abdominal pain and liver failure (Zain, 2011), with documented fatality rates as high as 40% (CDC, 2004; Wild & Gong, 2010).

In Ethiopia, previous studies reported high aflatoxin levels in certain cereals (maize, wheat, teff and sorghum) that are potentially used for the production of community based complementary foods (CFs). Chala *et al*. (2014) reported high aflatoxin content in sorghum. A study by Ayalew *et al*. (2006) reported the presence of aflatoxin in barley, sorghum, teff, and wheat samples. A study by Ayalew (2010) also reported aflatoxin in maize samples.

The control of aflatoxin contamination in agricultural crops starts at the field with implementation of good agricultural practices (GAPs). Pre-harvest practices such as ploughing, crop rotation, removing debris, row planting and others are important GAPs in preventing aflatoxin contamination in the field (Murphy *et al*., 2006). Good harvesting practices like harvesting at maturity, good storage practices and good manufacturing practices (GMPs) are also important in preventing aflatoxin contamination along the food chain (CAC, 2012; Reddy *et al*., 2010). Post-harvest practices like drying, sorting, and removal of moldy foodstuff are also important practices used to prevent aflatoxin contamination (Matumba *et al*., 2015).

However, effective implementation of the above strategies by the general public requires education and awareness about the health impacts of aflatoxins, and behavioural changes in pre- and post-harvest handling practices. Therefore, the purpose of this study was to assess the knowledge and practices of the mothers/caregivers of young children in selected areas in Ethiopia on issues related to mold and aflatoxin contamination in community based CFs for the purpose of prevention and management. To the best of our knowledge, this is the first study that assesses knowledge and practices of mothers/caregivers from farm-to-table on mold and aflatoxin contamination in East Africa.

3.3 Scope of the knowledge and practice assessment

The scope for the knowledge and practice assessment for preventing of CFs produced at community level from the risks of mold and aflatoxin contamination included:

- ✓ Assessment of the socio-demographic information of the mothers /caregivers,
- ✓ Assessment of the pre-harvest practices of people in the study area to collect information on the existing practices of plowing, crop rotation, removing debris and others.
- ✓ Assessment of the current harvest practices of people in the study area to collect information about harvesting time of the crops, the drying mechanisms, threshing methods, and other practices that have a direct effect on mold contamination.
- ✓ Assessment of the current storage practices of grains and legumes in the study areas to get information about storage types and facilities, cleaning methods, decontamination methods of mold infected grains, and others.
- ✓ Assessment of CF processing and handling at grain banks (GBs) to get information about women's knowledge, and the existing practices on CF ingredients collection and receiving, selection criteria, methods of preparing CF, storage practices, milling methods, personal hygiene, cleaning program at the grain bank, and others.

- ✓ Assessment of the existing practices of CF processing and handling at households (HHs) to get information about women or care-givers knowledge and practice on selection of CFs ingredients, sorting out mechanisms and criteria, the health impact of moldy foods, CFs processing methods, personal hygiene, and others.

3.4 Objective

The objective of this study was to assess the knowledge and practice of mothers/caregivers of young children to prevent the CFs from mold and aflatoxin contamination.

3.5 Materials and methods

3.5.1 Study areas and design

The study was conducted in 20 Woredas (Districts) in Amhara, Tigray, Oromia, and SNNP regional states of Ethiopia (Figure 3.1). In each region, there are five Districts. In each District, one semi-urban and nine rural kebeles (villages- smallest administrative unit), a total of ten villages were chosen for the knowledge and practice assessment. The study employed a cross-sectional quantitative survey of mothers/caregivers of young children (ages approximately 6 to 23 months). The source population for this study was all mothers/caregivers of young children from the 20 Districts across all regions.

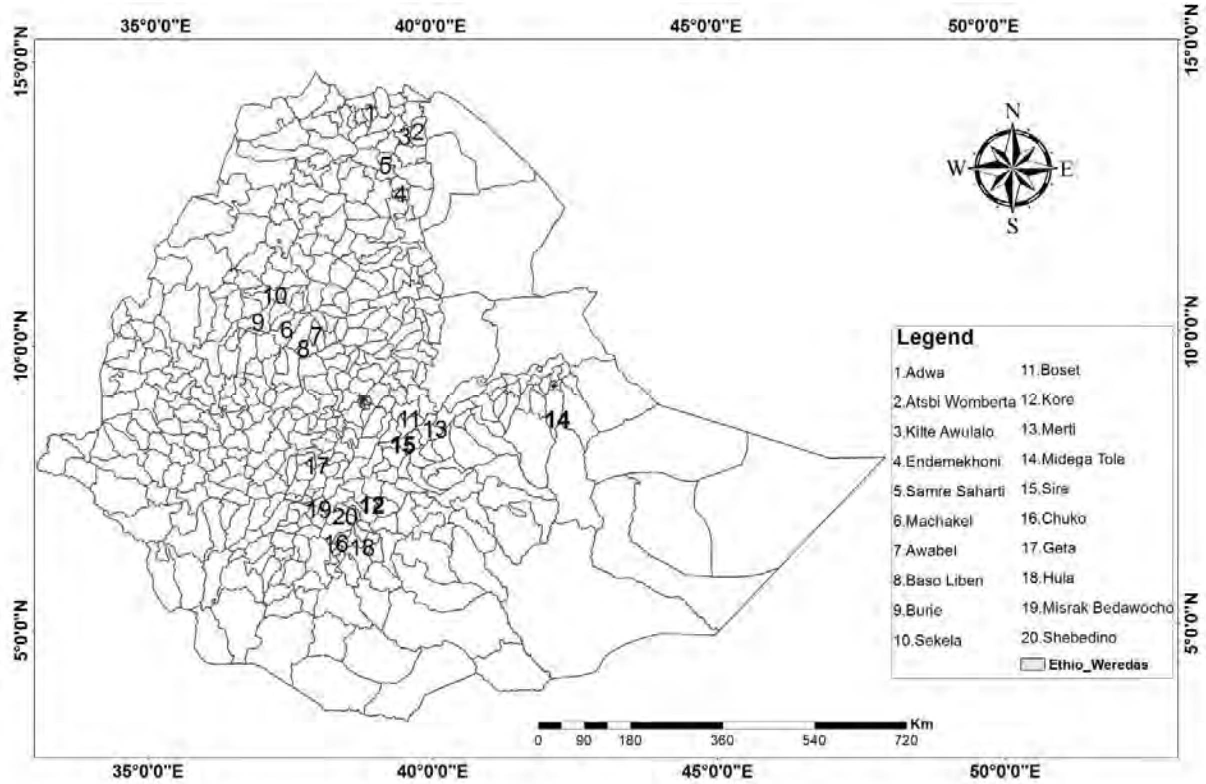

Figure 3.1 The 20 Districts of Ethiopia where the knowledge and practice assessment was conducted

3.5.2 Sampling technique

The Districts and the respective villages in each region, were selected by the Ethiopian Orthodox Church (EOC) and Research-inspired Policy and Practice Learning in Ethiopia (RiPPLE) with support of UNICEF- Ethiopia for the production of community based CFs. All the Districts and the respective villages were selected based on stunting prevalence and other factors. However, out of the 200 villages, 195 were involved during the knowledge and practice assessment because; five villages (two in Tigray and three in SNNP region) were not included due to some problems like inaccessibility of roads and other factors.

In each village, on average seven mothers have been selected by the village administrators and trained by the regional Universities about CFs processing and the GB (a house used to process and store CFs) practices. Hence, one mother from the seven trained mothers in each Village (a total of 195 mothers) was randomly selected for our knowledge and practice assessment.

3.5.3 Ethical consideration

Ethical approval and clearance for this study was obtained from the National Research Ethics Review Committee (NRERC) of the Ethiopian Ministry of Science and Technology and from Addis Ababa University (AAU), College of Natural Sciences Research Ethics Review Committee. And approval was also obtained from all regional health bureau review committee. The data were collected after explaining the purpose of the study to each study respondent and obtaining verbal informed consent.

3.5.4 Data collection

Quantitative data were collected using a structured questionnaire (Annex A). The questionnaires were organized into the following six sections: Socio-demographic information, pre-harvest knowledge and practices, harvest knowledge and practices, household storage knowledge and practices, and knowledge and practices of CFs processing and handling at GBs and HHs. The questionnaire was initially prepared in English language and later translated into local languages. The questionnaire was reviewed by micronutrient initiative (MI) program managers and various stakeholders and their comments were accommodated. Prior to data collection, the questionnaire was pretested using MSc and PhD students of the Center for Food Science and Nutrition (CFSN) and women volunteers. The data was collected at the mothers/caregivers' house with the help of health extension workers (HEWs) in each Village and EOC and RiPPLE Woreda coordinators. In the end, the questionnaires were gathered and checked for completeness by the principal investigator. The data were collected in December 2014 simultaneously across all the regions.

3.5.5 Data analysis

The data were entered into SPSS 20 statistical software. Then, the data were analyzed, presented and reported using descriptive statistics methods. Important indicators were computed for each region.

3.6 Results and Discussion

3.6.1 Socio-demographic information

Of the 195 children included in the study; 95 (49%) were between ages of 6-12 months, 67 (34%) were between ages of 13-18 months, 20 (10%) were between ages of 19-24 months, whereas 10 (5%) were older than 24 months of age. Of the 195 mothers, a total of 126 (65%) were between 20-30 years of age; 59 (30%) were between 31-40 years of age. Only very few mothers were above 41 years old. Figure 3.2 & Figure 3.3 shows the child's age and mother's age distribution respectively across all regions.

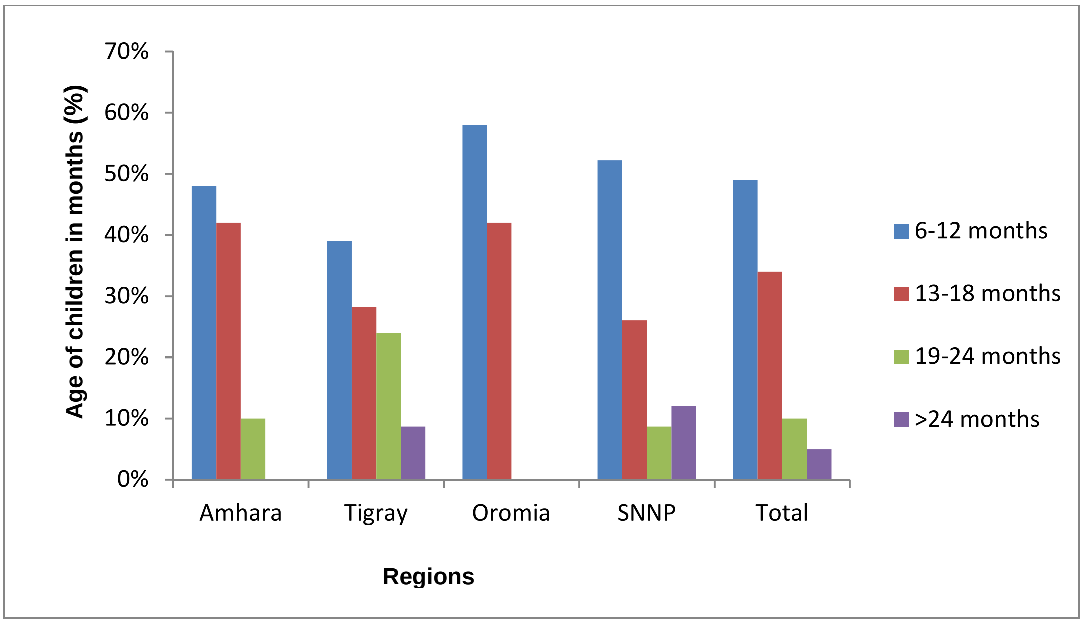

Figure 3.2 Proportion of age of children in months across all regions

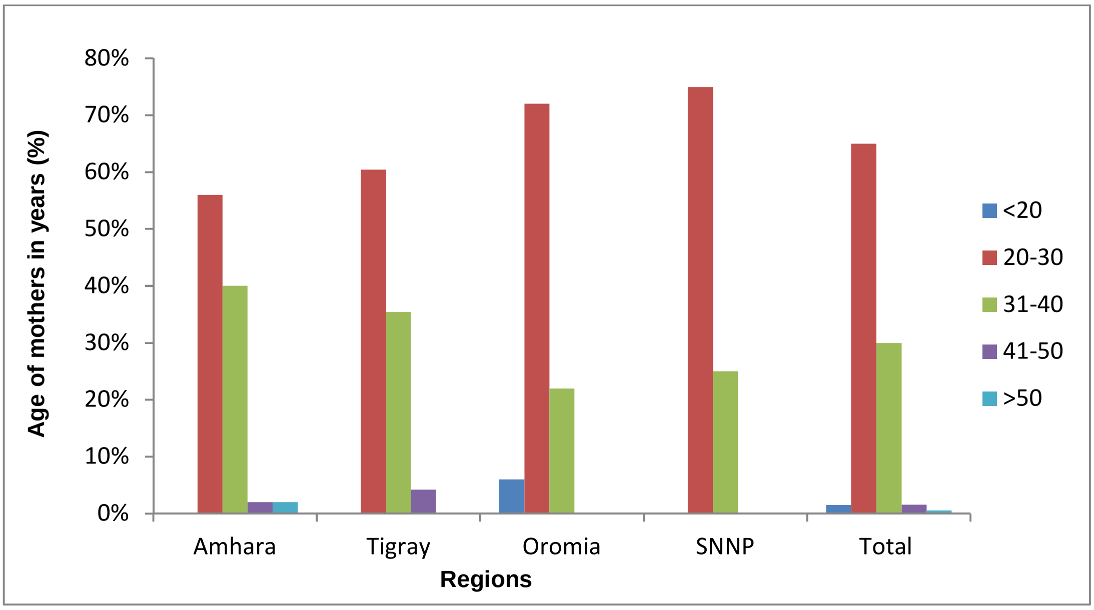

Figure 3.3 Proportion of age of mothers/caregivers in years across all regions

Among the 195 respondents, 82 (42%) were illiterate. Oromia (28 (56%)) and Amhara (27 (54%)) regions had the highest percentage of illiterate respondents. In contrast, 11 (23%) of the respondents in Tigray region had completed 9-12 grades, which is the highest of all regions.

3.6.2 Pre-harvest knowledge and practices

Of the 195 respondents, 186 (95%) had the knowledge and practice of ploughing the land before growing the next crop. Of all the respondents, 76 (39%) plough the land more than three times to plant cereals like; teff, maize, wheat or legumes like broad bean, pea and others. Among the four regions, Amhara, 40 (80%) was the highest in ploughing the land more than three times. The distributions of the frequency of ploughing across all regions are summarized in Figure 3.4.

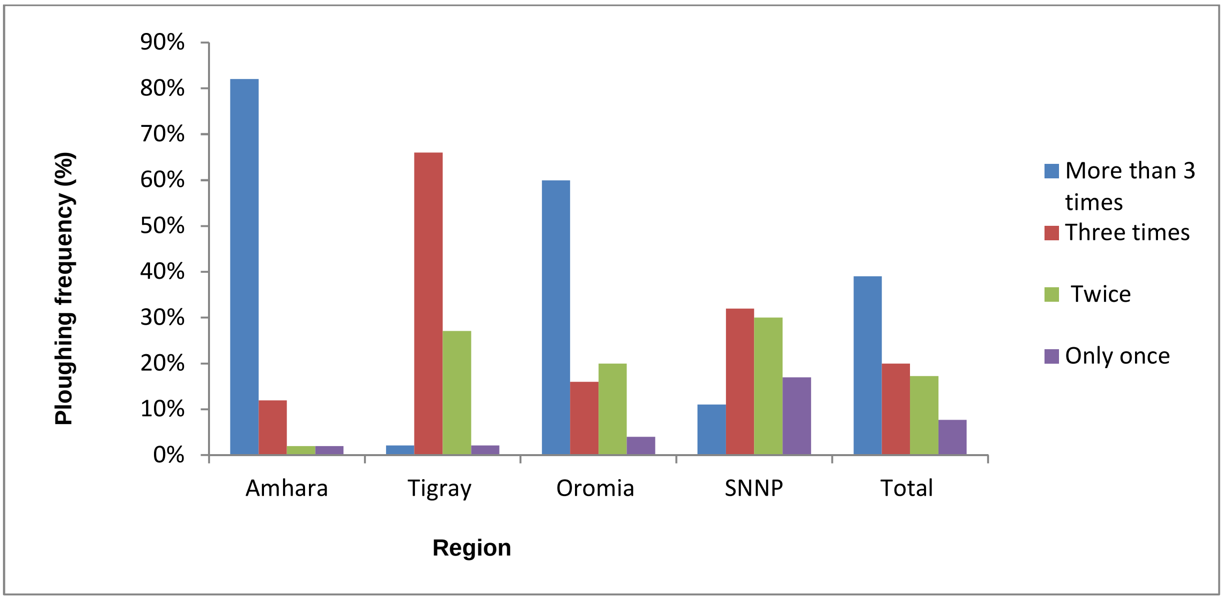

Figure 3.4 The frequency of ploughing across all regions

Similarly, 177 (91%) of the respondents were using the crop rotation schedule to avoid growing the same crop for consecutive years in the given field. Relatively, Tigray region was the highest (47 (98%)), while Oromia was the lowest (40 (80%)) in practicing crop rotation schedule. This result is supported by previous studies by Hell & Mutegi (2011) who reported that practices like crop rotation, well-timed planting, weed control, pest control and others minimized aflatoxin contamination at the field.

Of all the respondents, 157 (81%) of them reported the good knowledge and practice of removing old seed heads, stalks, and other debris which may serve as substrates for the growth of aflatoxin producing fungi. Among the four regions, Tigray had the highest percentage of best practices of removing old seed heads, stalks, and other debris while Oromia was the least performing region. Removing old seed heads, stalks, and other debris that may potentially serve as substrates for the growth of mycotoxin-producing fungi are the recommended practices to prevent mycotoxin contamination in the field (CAC, 2012). Hence, removing old seed heads, stalks, and other debris, need to be encouraged in the study areas. Figure 3.5 & Figure 3.6 shows the crop rotation practices and removing old seed heads, stalks, and other debris respectively across all regions.

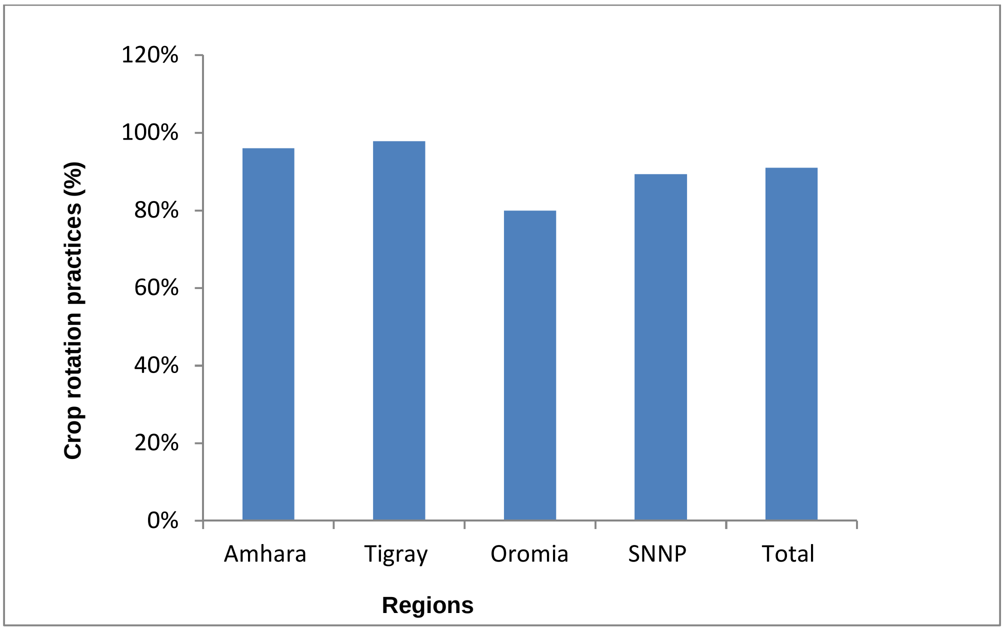

Figure 3.5 Crop rotation practices across all regions

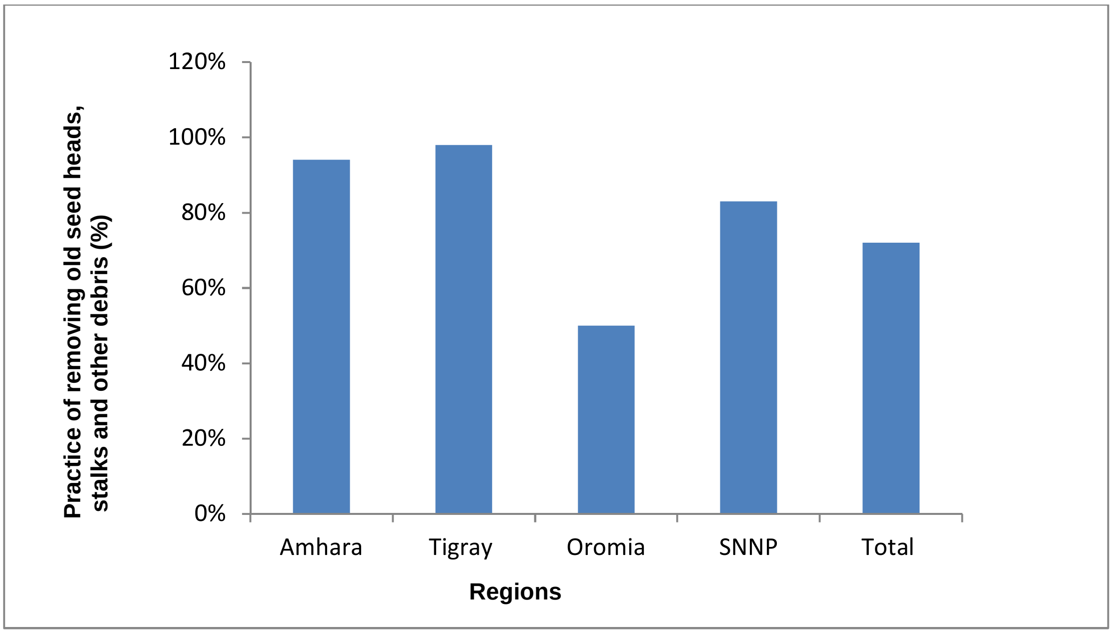

Figure 3.6 The practice of removing old seed heads, stalks, and other debris across all regions

Similarly, most of the respondents have the knowledge of climatic conditions that affect mold growth and aflatoxin contamination in cereals and legumes. Maize, wheat, and teff are the most common cereals while, broad bean, pea, chick pea, and haricot bean are the common legumes used for the preparation of CFs in the study arras. Of all the respondents, 86 (44%) of them answered that the rainy and humid climatic condition was critical for mold growth. A total of 52 (27%) respondents reported that just rain was the most critical condition. Indeed, previous studies by Algul & Kara (2014) reported the impacts of humidity and temperature on mold growth and aflatoxin contamination in cereals. Thomson & Henke (2000) and Alborch *et al*. (2011) reported that crops in tropical and subtropical areas with high humidity and temperature are susceptible to mold growth and aflatoxin contamination. Similarly, a case study in Malaysia showed that stored crops at high temperatures (28 to 31 ^{0}C) and at high relative humidity (70 to 80%) were easily contaminated by mycotoxin-producing fungi (Afsah-Hejri *et al*., 2013).

3.6.3 Harvest knowledge and practices

The respondents were asked about the different harvest knowledge and practices that contribute to mold spoilage and aflatoxin contamination of cereals and legumes. The majority of the respondents (185 (95%)) were harvesting as soon as the crops were matured. When the crop is harvested immediately at maturity, mold and aflatoxin contamination decreases significantly along the food chain. Previous study by Hell & Mutegi (2011) also reported that harvested immediately at maturity prevents aflatoxin contamination. Concerning the methods of checking for maturity of the crops, almost half of the respondents (107 (55%)) assess this based on color of the crop. Hence, the result indicates that the respondents have the knowledge to check the maturity of the crops.

Concerning the threshing methods, the majority of the respondents (138 (71%)) used trampling by hoofed animals usually cattle: letting them to walk over the harvested crops on a threshing bare floor with smeared cow dung. Only 57 (29%) of the respondents used manual (beating with a stick) threshing methods. Hence this result indicates that the respondents were not aware of the cross contamination from the threshing floor.

After threshing, the respondents were asked whether they further decrease the moisture content of cereals and legumes before storage. Of all the respondents, 124 (64%) used the practice of drying to decrease the moisture content. Solar drying is the common method used in all regions. The majority of the respondents (134 (69%)) used solar drying on a bare ground, while 34 (17%) used solar drying using plastic sheets.

The result indicates that the majority of the respondents did not have the knowledge about the most appropriate drying mechanisms. Solar drying on a bare floor is susceptible to cross contamination by aflatoxigenic mold. Besides, the moisture level of cereals and legumes increases by absorbing moisture from the ground. Therefore, to reduce or prevent production of mycotoxins, drying should take place soon after harvest and as rapidly as feasible (Chulze, 2010), but drying on a bare ground should be avoided. However, checking the moisture level of cereals and legumes using traditional techniques like scratching by finger nails and breaking sound using teeth, and others should be encouraged.

Besides checking the moisture level of cereals and legumes after solar drying, the respondents had the practice of sorting out less quality cereals and legumes based on color, grain size, moldiness, mechanical damage and a combination of these criteria. This is a very good practice and should be encouraged since it is supported by previous study by Matumba *et al*. (2015) who reported that hand sorting had the greatest effect on mycotoxin removal in maize. Another study by Hell & Mutegi (2011) also reported that sorting and cleaning are important post harvest interventions to reduce aflatoxin contamination in key crops in Sub-Saharan Africa.

However, the sorted out (moldy) cereals and legumes are being given to animals. Of all the respondents, 56 (29%) of them had the practice of giving the sorted-out cereals and legumes to chicken and 21 (11%) used it as donkey feed. Hence, this result indicates that the respondents did not have the knowledge about the health impact of aflatoxin to animal’s health and, in turn human health due to carry over to meat and eggs.

3.6.4 Household storage knowledge and practices

The study respondents used traditional storage structures, mostly ‘Gota’, ‘Gotera’, polypropylene bags, and underground pit. An overview of the answers is shown in Table 3.1. ‘Gota’ is an indoor cylindrical grain storage structure made from a mixture of mud, cow dung and straw. It is built up of a series of segments made in the form of rings which are fitted one on top of the other and reassembled to pieces if needed (Bekele *et al*., 2006).

‘Gotera’ is a cylindrical storage structure made by interweaving vines, bamboo or other plant materials and plastered with mud and cow dung on the inside. It can mostly be kept outdoors in front of the main house and it stands on a stone (Bekele *et al*., 2006). While, underground pit is traditionally used in the lowland areas for the storage of sorghum under a reasonably airtight condition (Bekele *et al*., 2006). The traditional storage structures, ‘Gota’, ‘Gotera’ and underground pit are indicated respectively in Figure 3.5.

a) b) c)

Figure 3.7 Traditional storage structures for cereals a) ‘Gota’ b) ‘Gotera’ c) Underground pit

Out of 195 respondents, 53 (27%) used ‘Gota’, while 54 (28%) used a combination of both ‘Gota’, ‘Gotera’ and polypropylene bags as grain storage structures at their homes. However, few respondents (14 (7%)) used underground pit storage for sorghum.

Table 3.1 Household storage knowledge and practices relevant to aflatoxin contamination of cereals and legumes used for CFs production

No.	Factors	Regions				Total
		Amhara	Tigray	Oromia	SNNP	
1	**Traditional Storage structures used by the study respondents'**					
	✓ 'Gota'	64% (32)	33% (16)	10% (5)	0% (0)	27% (53)
	✓ 'Gotera'	0% (0)	4% (2)	0% (0)	53% (25)	14% (27)
	✓ Underground pit	0% (0)	0% (0)	28% (14)	0% (0)	7% (14)
	✓ Polypropylene bags	10% (5)	19% (9)	20% (10)	47% (22)	24% (46)
	✓ Modern silos	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)
	✓ Gota/Gotera and polypropylene bags	24% 12)	44% (21)	42% (21)	0% (0)	28% (54)
2	**Reason for not using modern storage structures like silos and others to prevent aflatoxin contamination in the stored cereals**					
	✓ Costly	20% 10)	42% (20)	2% (1)	34% (16)	24% (47)
	✓ Not available	74% 37)	56% (27)	96% (48)	19% (9)	62% (121)
	✓ No need	4% (2)	2% (1)	0% (0)	21% (10)	7% (13)
	✓ Lack of information	0% (0)	0% (0)	2% (1)	23% (11)	6% (12)
3	**Practice of removing previously stored cereals and legumes before new storage to prevent aflatoxin contamination**	86% (43)	75% (36)	100% (50)	96% (45)	89% (174)
4	**Practice of disinfecting storage structures to prevent aflatoxin contamination**	84% (42)	100% (48)	100% (50)	94% (44)	94% (184)
	✓ Smearing with cow dung	78% (39)	23% (11)	67% (32)	32% (15)	50% (97)
	✓ Smearing with ash	6% (3)	47% (22)	25% (12)	34% (16)	27% (53)
	✓ Smoking with 'woira' plant leaves	2% (1)	6% (3)	2% (1)	6% (3)	4% (8)
	✓ Neem tree leaves	0% (0)	11% (5)	0% (0)	2% (1)	3% (6)
	✓ Insecticide fumigation	0% (0)	0% (0)	6% (3)	0% (0)	2% (3)

The result indicates that respondents had no knowledge about the need to use modern silos (0% used modern silos). Since traditional storage structures are more susceptible to mold spoilage and aflatoxin contamination, pest and rodents' infestations. A study by Dubale *et al*. (2014) reported that traditional storage structures increase the deterioration of maize grain by aflatoxigenic mold. Another study by Ayalew *et al*. (2006) reported that underground pit storage of sorghum is more susceptible to mold spoilage. A study by Dejene *et al*. (2004) also reported the incidence of maximum mold contamination in sorghum stored for about 24 months in underground pit. Another study by Hell *et al*. (2000) also reported that traditional storage structures in Benin, West Africa are susceptible to aflatoxin contamination. These traditional storage structures are exposed to heat, high humidity, lack of aeration in the stores, and insect and rodent damage resulting in mold spoilage and aflatoxin contamination. Hence, traditional storage structures in the study areas need to be improved.

Concerning the practice of cleaning the storage structures, a large number of respondents (184 (94%)) clean and disinfect the storage area before storage of cereals and legumes to prevent aflatoxin contamination. Besides, large number of the respondents (174 (89%)) had the knowledge and practice of removing previously stored cereals and legumes before storing new ones. To clean storage structures, the majority used smearing with cow dung (97 (50%)), smearing with ash (53 (27%)), and very few used smoking with African Olive (Olea Europea subspecies Africana) or 'woira' (8 (4%)) and neem (Azadirachta indica) tree leaves (6 (3%)). A study by Mahmoud *et al*. (2011) reported an antifungal activity of neem leaves extract. Hence using neem tree leaves as well as smoking needs to be encouraged.

3.6.5 CFs processing and handling knowledge and practices at grain banks

During the knowledge and practice survey the GB was functional only in Amhara and Tigray regions. As a result, the data was from these two regions (Table 3.2). A total of 98 respondents, 50 from Amhara and 48 from Tigray were involved and the percentage was calculated out of 98. The majority 80 (82%) of the respondents obtained the CFs ingredients from their own agricultural produce. Only very few obtained from GBs and local markets.

Almost all the respondents had the knowledge and practice of the processing methods of CFs at the GB. They used the following CFs processing steps accordingly; cleaning, soaking, de-hulling, drying, roasting, milling, sifting and packaging. But, the majority (89 (91%)) of the respondents erroneously believes that roasting can decontaminate the toxins produced from moldy cereals and legumes. However, a study by Bullerman & Bianchini (2007) reported that high temperature (above 150 ^{0}C) processes cause varying degrees of reduction of mycotoxin concentrations, but most mycotoxins are moderately stable in most food processing systems. A study by Yazdanpanah *et al*. (2005) also reported that roasting pistachio nuts showed some degree of aflatoxins degradation (ranging from 17% to 63%).

Table 3.2 Respondents knowledge and practices that may contribute to aflatoxin contamination of CFs and their ingredients at grain banks in Amhara and Tigray regions

Factors	Regions		
	Amhara	Tigray	Total
1. Source of ingredients for CFs preparation			
✓ Own agriculture	92% (46)	71% (34)	81% (80)
✓ Grain banks	2% (1)	15% (7)	8% (8)
✓ Local markets	6% (3)	8% (4)	7% (7)
✓ Donors	0% (0)	0% (0)	0% (0)
2. Qualities for selecting CF ingredients			
✓ Color	0% (0)	4% (2)	2% (2)
✓ Type	0% (0)	2% (1)	1% (1)
✓ Insect infestations	0% (0)	0% (0)	0% (0)
✓ Odor	0% (0)	2% (1)	1% (1)
✓ Any two criteria	0% (0)	15% (7)	7% (7)
✓ Any three criteria	2% (1)	15% (7)	8% (8)
✓ Any four criteria	96% (48)	29% (14)	63% (62)
3. Fate of sorted out cereals and legumes			
✓ Discard	68% (34)	77% (37)	72% (71)
✓ Sell	32% (16)	12% (6)	22% (22)
4. Did you mix moldy cereals and legumes with non-moldy ones while preparing CFs	8% (4)	29% (14)	18% (18)
5. Do you think that roasting decontaminates the toxins produced from moldy cereals and legumes	44% (22)	79% (38)	61% (60)
6. Milling type used in grain bank			
✓ Dry milling	100% (50)	100% (48)	100% (98)
✓ Wet milling	0% (0)	0% (0)	0% (0)
7. Cleaning the miller between batches			
✓ Yes	92% (46)	75% (36)	84% (82)
✓ No	8% (4)	25% (12)	16% (16)
8. Duration of CF storage at the grain bank			
✓ One week	2% (1)	2% (1)	2% (2)
✓ Three weeks	2% (1)	2% (1)	2% (2)
✓ One month	96% (48)	96% (48)	98% (96)
9. Cleaning program at the grain bank facility			
✓ Yes	34% (17)	34% (17)	34% (34)
✓ No	66% (33)	66% (33)	67% (66)

At the GB, dry milling was used exclusively (98 (100%)) and they reported that, the mill was cleaned between each batch of milling to avoid cross contamination between milling. However, more respondents 33 (34%) did not clean the GB facility, only 17 (17%) reported to clean the GB facility frequently. This result indicates that mothers involved in CFs processing at the GB did not have the knowledge about food service sanitation. A study by Todd *et al*. (2007) reported that good personal hygiene and sanitary handling practices in the food processing area is essential to ensure food safety. Hence, the sanitary condition of the GBs needs to be improved to prevent the CFs and their ingredients from the risks of mold and aflatoxin contamination.

3.6.6 CFs processing and handling knowledge and practices at households

A total of 137 (70%) respondents obtained ingredients used for CFs preparation from their own agricultural produce, only very few respondents (13 (7%)) used the local market. About 50 (26%) respondents used color, odor, type, moldiness, and insect infested criteria to select CFs ingredients. Similarly, a large number of respondents (164 (84%)) had the knowledge and practice of sorting out of mechanically damaged cereals and legumes while preparing CFs. This is a very good practice since mechanically damaged cereals and legumes might be contaminated by mold and aflatoxin. Hence sorting out mechanically damaged cereals and legumes need to be encouraged. Besides, our results are consistent with previous study by Matumba *et al*. (2015) who reported a manual removal of immature, broken and discoloured grains, although a tedious task, can significantly remove mycotoxins with an efficiency of 95% or higher in contaminated white maize. An overview of the respondents' knowledge and practices at households that may contribute to aflatoxin contamination of CFs and its ingredients is depicted in Table 3.3.

Table 3.3 Respondents' knowledge and practices that may contribute to aflatoxin contamination of CFs and their ingredients at households

No	Factors	Regions				Total
		Amhara	Tigray	Oromia	SNNP	
1	**Source of ingredients for CFs preparation**					
	✓ Own agricultural produce	90% (45)	58% (28)	76% (38)	55% (26)	70% (137)
	✓ Local market	8% (4)	4% (2)	2% (1)	13% (6)	7% (13)
	✓ Own and market	2% (1)	35% (17)	22% (11)	32% (15)	23% (44)
	✓ *Safety nets	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)
	✓ Donors aid	0% (0)	0% (0)	0% (0)	0% (0)	0% (0)
2	**Selection criteria of cereals and legumes for CFs preparation**					
	✓ Color	0% (0)	4% (2)	0% (0)	9% (4)	3% (6)
	✓ Type	0% (0)	6% (3)	12% (6)	34% (16)	13% (25)
	✓ Insect infested	0% (0)	6% (3)	0% (0)	11% (5)	4% (8)
	✓ Moldiness	0% (0)	0% (0)	0% (0)	4% (2)	1% (2)
	✓ Odor	0% (0)	0% (0)	45% (22)	0% (0)	11% (22)
	✓ Any two criteria	2% (1)	44% (21)	8% (4)	4% (2)	15% (28)
	✓ Any three criteria	2% (1)	35% (17)	8% (4)	23% (11)	17% (33)
	✓ Any four criteria	0% (0)	0% (0)	2% (1)	15% (7)	4% (8)
	✓ All criteria	96% (48)	4% (2)	0% (0)	0% (0)	26% (50)
3	**What if when observe mold in foods prepared for child or family**					
	✓ Discarded	84% (42)	21% (10)	16% (8)	15% (7)	34% (67)
	✓ Feed to animals	28% (14)	63% (30)	18% (9)	53% (25)	40% (78)
	✓ Sell	62% (31)	0% (0)	0% (0)	6% (3)	17% (34)
	✓ Eat by processing (roasting etc.)	2% (1)	10% (5)	2% (1)	0% (0)	4% (7)
4	**Using moldy cereals for brewing local beverages like 'tella'**	34% (17)	23% (11)	26% (13)	8.5% (4)	23% (45)
5	**Knowing the health effect of local beverages made from moldy cereals**	6% (3)	29% (14)	8% (4)	11% (5)	14% (26)
6	**Drinking local beverages made from moldy cereals at times of lactation**	42% (21)	13% (6)	10% (5)	0% (0)	16% (32)
7	**Sorting out of mechanically damaged cereals and legumes**	96% (48)	98% (47)	78% (39)	64% (30)	84% (164)
8	**The importance of sorting out mechanically damaged cereals and legumes**					
	✓ Susceptibility to mold spoilage	4% (2)	17% (8)	0% (0)	60% (28)	20% (38)
	✓ Decrease of nutritional quality	4% (2)	4% (2)	42% (21)	4% (2)	14% (27)
	✓ Decrease of sensory quality	52% (26)	15% (7)	4% (2)	11% (5)	20% (40)
	✓ Both mold spoilage and nutritional loss	2% (1)	42% (20)	2% (1)	0% (0)	11% (22)
	✓ Both Nutritional and sensory loss	30% (15)	2% (1)	30 (15)	0% (0)	16% (31)
	✓ All (mold, sensory and nutritional loss)	8% (4)	19% (9)	0% (0)	0% (0)	7% (13)

*__Safety net__: Provision of food commodities (like wheat, maize, etc.) as a payment for labour intensive activities.

Observing mold in foods prepared for a child or a family was common among the respondents across all regions. Out of 195 respondents, 161 (83%) of them observed mold in children and/or family foods. Respondents were asked how they deal with moldy foods. Nearly half of the respondents (78 (40%)) said they had to feed the moldy foods to animals, a third of the respondents (67 (34%)) had the practice of discarding it, 34 (17%) had the practice of selling it, while 7 (4%)

had eaten the food themselves after processing. The result indicates that most of the respondents did not have the knowledge about the health impacts of aflatoxin to humans and animals.

However, our results are consistent with a previous study by Matumba *et al*. (2016) who reported that the people in Malawi especially females do not have the knowledge about the health implications associated with moldy food- and feedstuffs. Another study by Sanders *et al*. (2015) reported that the Flemish population lacked awareness about the toxic effects of mycotoxins. Mboya & Kolanisi (2014) also reported that people in Tanzania used fungal infected staples for food, implying that people are not fully aware of health hazards associated with the ingestion of mycotoxins. A similar study by Ezekiel *et al*. (2013) in Nigeria reported that about 85% of peanut cake consumers did not have awareness about the health impacts of aflatoxin. A study by Awuah *et al*. (2008) also reported that people in Ghana are unaware of the harmful effects of aflatoxins. Hence, sensitisation of the people concerning the health impacts of aflatoxin needs to be urgently implemented.

3.7 Conclusion

A knowledge and practice study was carried out in Ethiopia that addressed a range of issues used to prevent aflatoxin contamination from farm-to-table. Respondents had good knowledge and practice of GAPs like ploughing, crop rotation, and removing old seed heads, stalks, and other debris. The most common threshing methods used were trampling by hoofed animals on a threshing floor, which is susceptible to contamination by animal’s excrements and other microorganisms. Most of the study respondents check the required moisture level of cereals and legumes using traditional techniques like scratching by finger nails and breaking sound using teeth before storage. The common storage methods used were ‘Gota’, ‘Gotera’ and underground pit; which are susceptible to mold spoilage and aflatoxin contamination. Mold spoiled cereals and legumes were used as an animal feed, especially to chicken and donkey. Further, moldy cereals were used to prepare local alcoholic beverages. Hence, the respondents were unaware of the toxic effects of aflatoxin contaminated foods and/or feeds to human and animal health. Therefore, more information needs to be disseminated/ provided to the communities on how to handle moldy foods and feeds, and measures could be taken to eliminate or reduce aflatoxin exposures.

CHAPTER 4. EVALUATION OF THE MICROBIOLOGICAL SAFETY (*CRONOBACTER SAKAZAKII*, COLIFORMS AND *ESCHERICHIA COLI*) OF COMPLEMENTARY FOODS PRODUCED AT COMMUNITY LEVELS USING LOCALLY AVAILABLE INGREDIENTS IN ETHIOPIA

4.1 Abstract

Cronobacter sakazakii, coliforms, *E. coli* and other food borne microbial agents have caused foodborne illnesses in young children through consumption of a variety of foods, including complementary foods (CFs). The incidence of *C. sakazakii*, coliforms and *E. coli* inCFs and their ingredients were studied. A total of 146 samples collected from 20 Districts from Amhara, Tigray, Oromia and SNNP regions were tested for the presence of *C. sakazakii*, coliforms and *E. coli*. The incidence of *C. sakazakii* was detected using ISO/TS 22964:2006 method. Coliforms and *E. coli* were detected using the conventional most probable number (MPN) method. *C. sakazakii* was detected from 3/20 (15%) moldy cereals and legumes, 10/66 (15%) pre-milling cereals and legumes, 1/20 (5%) post production CFs, 1/20 (5%) CFs (HH), and 2/20 (10%) CFs (GB). Coliforms were detected from 12/20 (60%) moldy cereals and legumes up to a maximum of >1100 CFU/g, 9/66 (13.6%) pre-milling cereals and legumes up to a maximum of >1100 CFU/g, 13/20 (65%) post production CFs up to a maximum of 460 CFU/g, 16/20 (80%) CFs (HH) up to a maximum of 1100 CFU/g, and 16/20 (80%) CFs (GB) up to a maximum of 150 CFU/g. *E. coli* was not detected from any of the pre-milling cereals and legumes. But it was detected from 3/20 (15%) moldy cereals and legumes up to a maximum of 23 CFU/g, 1/20 (5%) post production CFs at a level of 4 CFU/g, 3/20 (15%) CFs (HH) up to a maximum of 150 CFU/g, and 2/20 (10%) CFs (GB) up to a maximum of 9 CFU/g. Over all, *C. sakazakii* was detected from pre-milling cereals and legumes, moldy and CFs samples. The percentage incidences of coliforms and *E. coli* were higher in post production CFs and one month stored CFs indicating poor hygienic practices both at HHs and GBs. Hence, the hygienic practices both at HHs and GBs need to be improved.

Keywords: *C. sakazakii*; Coliforms; *E. coli*; Complementary foods; Cereals; Legumes; Households; Grain banks.

4.2 Introduction

Ethiopia facing the conventional burdens of stunting in under-five children. The CSA (2014) reported 40% stunting prevalence among children less than 5 years of age. Such a high prevalence of undernutrition reaches its peak during the the period when CFs are often introduced, this may be explained, in part by inadequacies in CFs perhaps complementary feeding practices. UNICEF Ethiopia and partners, recognizing this problem, developed a project for the local production of CFs that would optimize the nutritional intake, and thus nutritional status of children 6-23 months in rural settings (models) of Amhara, Tigray, Oromia and Southern Nations Nationalities and Peoples (SNNP) regions.

The project in the rural setting organizes the community to initiate and implement a centralized production and distribution of CFs through a bartering system. The project provides limited equipment (storage bins, roasting pans, bags, balance and sealer) and most of the raw products (cereals/legumes) are contributed by the community, depending on their availability. These are stored in a GB. Women are identified and trained to process the CFs by following a standard formulation developed by the Center for Food Science and Nutrition, AAU. The bartering system invites mothers to bring 2 kg of raw materials (cereals/legumes) and receives 3 kg of prepared CFs in return.

The project has been well integrated and accepted by the communities involved in the pilot. However, the CFs and their ingredients might be a risk of contamination by food borne microbial agents such as *C. sakazakii*, coliforms, *E. coli*, and others and the preventive measures taken to reduce this risk is not properly stated perhaps not properly managed. Since, foodborne microbial agents can cause diarrhoeal diseases and ill-health in infants (Motarjemi, 2000). Globally, diarrhoea is the second leading cause of death for children under the age of five (Black *et al*., 2003) and is a leading cause of growth faltering and malnutrition. A pronounced proportion of diarrhoeal incidences occur due to foodborne pathogens transmitted by unhygienic preparation of foods in households (Sheth & Dwivedi, 2006).

C. sakazakii, is a motile, non-spore forming, Gram-negative rod belonging to the family Enterobacteriaceae. It could be isolated from a wide spectrum of food and food ingredients (Beuchat *et al*., 2009; Chap *et al*., 2009; Turcovsky *et al*., 2011; Vojkovska *et al*., 2016). It was isolated from plant food and food ingredients like cereal, fruit and vegetables, legume products, herbs and spices as well as from animal food sources like milk, meat and fish products. The spectrum of *C. sakazakii* contaminated food covers both raw and processed food. Fresh, frozen, ready-to-eat, fermented and cooked food products as well as beverages and water were found to be contaminated by *C. Sakazakii* (Friedmann, 2007; Beuchat *et al*., 2009). However, powdered milk infant formula (PIF) and milk powder have been the main sources and the vehicles responsible for 50-80% of *C. sakazakii* infections (Mullane *et al*., 2007).

C. sakazakii has caused food borne illnesses through consumption of a variety of foods, including infant foods (Shaker *et al*., 2007). This bacterium is an emerging opportunistic pathogen that is associated with rare but life-threatening cases of meningitis, necrotizing enterocolitis, and sepsis in premature and full-term infants (Lou *et al*., 2014). Infants aged <28 days are considered to be most at risk.

Coliforms are rod shaped gram negative, non-spore forming and motile/non-motile bacteria which can ferment lactose with the production of acid and gas when incubated at 36 ^{0}C. *E. coli* is the major species in the coliform group. Coliforms and *E. coli* are referred to as indicator organisms since their presence is used to indicate the possible incidence of pathogens in foods. Coliforms and *E. coli* detection has been adopted by world-class food industry to determine microbiological quality of food products. Detection of *E. coli* suggests contaminations from direct or indirect fecal origins of humans and warm-blooded animals. High counts of *E. coli* and coliform in food samples directly imply poor practices in food handling and production operations in the manufacturing chain (de Sousa *et al*., 2002). As a result, coliforms and *E. coli* enumeration are used as a food quality parameter (Gonzalez et *al*., 2003).

Coliforms and *E. coli* have caused food borne infection through consumption of various types of foods, including; fruits, vegetables, milk and milk products, fish and fish products, meat and meat products, CFs, and others. Consumption of contaminated CFs with *E. coli* appeared to be associated with a higher frequency of diarrhoea and malnutrition in children. A study by Islam *et al*. (2012) reported that around 40% of CFs given to the children was contaminated with a higher number of coliform bacteria ($\geq$ 100 CFU/g of food) and *E. coli*, which is an indicative of direct faecal pollution. A number of pathogenic types, including the enterotoxigenic *E. coli*, enteropathogenic *E. coli* and Shiga toxin-producing *E. coli* were detected from the isolated *E. coli* (Islam *et al*., 2012). A study by Kung'u *et al*. (2009) also reported that higher number of coliforms was detected from locally prepared CFs in Tanzania.

The CFs made from locally available ingredients in developing countries like Ethiopia has the possibility of contamination by *C. sakazakii*, coliforms and *E. coli*. Since cereals and legumes, which are ingredients to CFs are trashed using trampling by hoofed animals usually cattle, letting them to walk over the harvested grains on a threshing bare floor with smeared cow dung's. In addition, there is high possibility of cross contamination through the food chain due to poor practices in food handling and production operations. Therefore, evaluating the safety of the locally prepared CFs in terms of *C. sakazakii*, coliforms and *E. coli* are paramount importance to safeguard the health of young children in the study areas and to provide evidence for safety interventions.

4.3 Objective

4.3.1 General objective

To evaluate the safety of complementary foods from microbiological contaminations; *C. sakazakii*, coliforms and *E. coli* produced at community level using locally available ingredients.

4.3.2 Specific Objectives

- Determine the contents of *C. sakazakii*, coliforms and *E. coli* in moldy cereals and legumes purposively removed during CFs preparation,
- Determine the contents of *C. sakazakii*, coliforms and *E. coli* in the pre-milling ingredients used for CFs preparation,

- Determine the contents of *C. sakazakii*, coliforms and *E. coli* in post production CFs and one month stored CFs at households (HHs) and GBs,
- Compare the incidences of *C. sakazakii*, coliforms and *E. coli* in the CFs stored for one month at HHs and GBs.

4.4 Materials and Methods

4.4.1 Sampling areas

The samples were collected from two rural piloting villages in each piloting District in Amhara, Tigray, Oromia, and SNNP regional states of Ethiopia as indicated in Table 4.1. The two villages were randomly selected out of the nine villages based on the bartering performance (i.e. the number of times by which they prepared CFs and distribute to mothers) and the total number of mothers that have been taken the CFs for the last three months. The selection was done in consultation with the EOC and RiPPLE coordinators in each District across all regions. Because the coordinators in each District have all the records of each village GB activities.

Table 4.1 Villages in each District where samples were collected across all the regions

Region	District	Name of villages	Number of villages
Amhara	Baso Liben	Yelemlem and Limichm	2
	Awabel	Zinda and Wejel Ankirak	2
	Machkel	Kidamin Zuria and Yedefas	2
	Burie	Denbun and Fezel	2
	Sekela	GishAbay and Kolel	2
Tigray	Endemekhoni	Embahasti and Shimta	2
	Atsbi Womberta	Felege Weyni and Gebrekidan	2
	Kilte Awlalo	Debre-tsion and Negash	2
	Samre Saharti	Amdi-Weyane and May-tekli	2
	Adwa	Debre Genet and Weyenti	2
Oromia	Midega Tole	Berzala and Lencha	2
	Merti	Gora Silinko and Shemo Gado	2
	Sire	Alelu Gasala and Ibsata Duga	2
	Kore	Doda Deyyu and Bulchana Hulluko	2
	Boset	Dongorre Tiyo and Dongorre Furda	2
SNNP	Geta	Agata and S/Koreficha	2
	Misrak Badwacho	Anburse Anjulo and Kenchera	2
	Aleta Chuko	Chuko Lamala and Chuko Weyama	2
	Shebedino	Morocho Negash and Morocho Qutela	2
	Hula Tula	Finchawa and Tulo	2
		Total number of Villages	40

4.4.2 Sample collection

Four types of samples: 1) moldy cereals and legumes: purposively removed during the cleaning process due to mold (visually), insect or other contamination, 2) pre-milling: cleaned and roasted cereals and legumes– one sample from each type of cereal or legume that was used in CFs production, 3) post production CFs (no storage time following production), and 4) CFs stored in HHs (CFs(HHs)) and GBs (CFs(GBs)) for one month were collected from the two randomly selected rural Villages across all the regions. The flow diagram for CFs production at the GBs and sampling points is depicted in Figure 4.1.

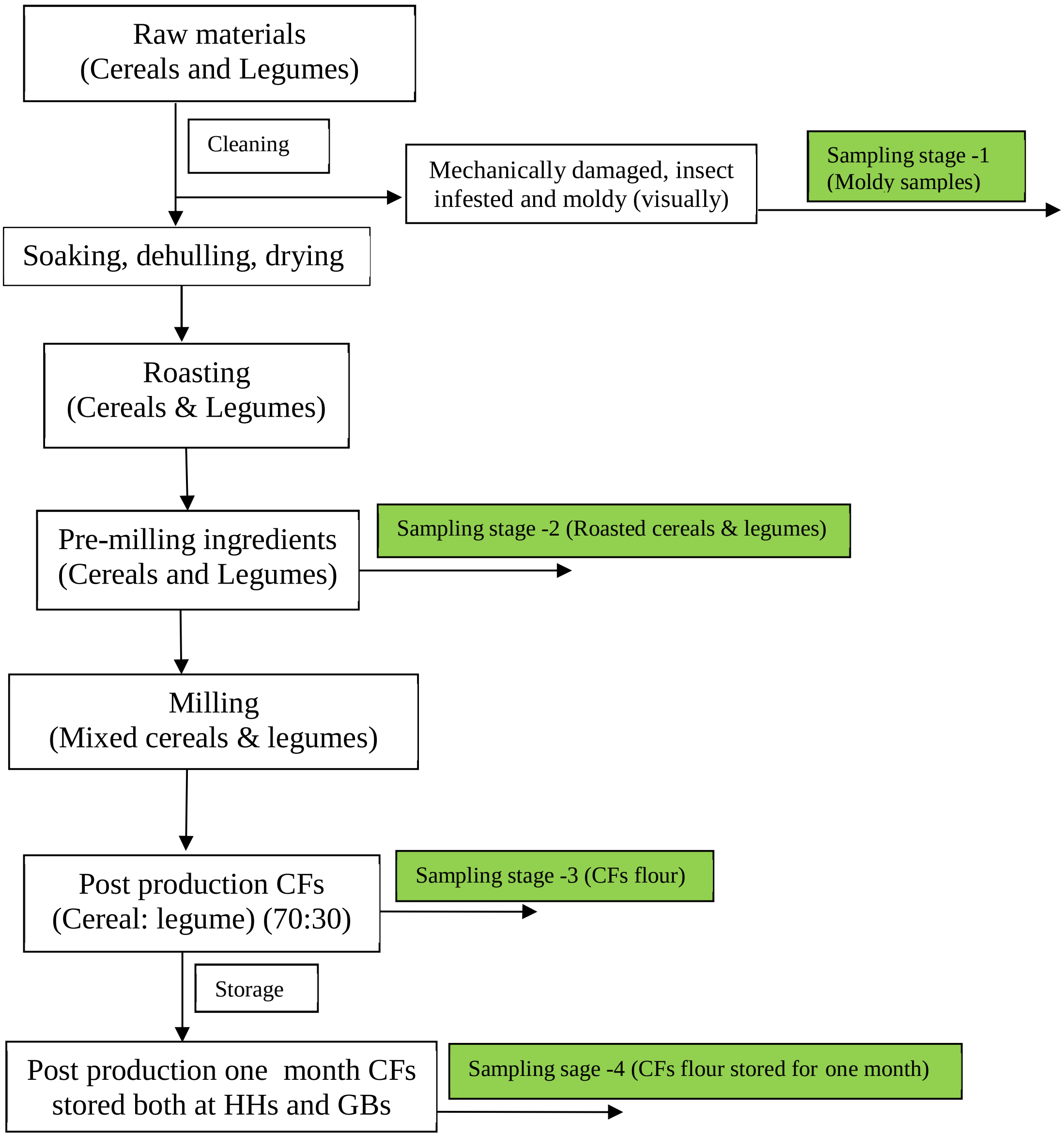

Figure 4.1 Flow diagram of complementary foods production at the GBs and HHs and sampling points

Mothers who had completed the training by the regional University were in charge of processing CFs across all the piloting Villages GBs. The training given to mothers were basically focused on the processing steps of cereal based CFs at the community level and the importance of CFs for the young children. The trained mothers were involved in: receiving the raw materials, cleaning, roasting, milling and other activities during CFs preparation with close supervision by HEWs.

1) Sampling stage-1 (Moldy cereals and/or legumes):

Moldy samples included cereals and/or legumes that were moldy (visually), mechanical damaged and/or insect infested; these were the sorted-out cereals and legumes during cleaning at each GB. These samples were collected at the GBs just prior to roasting. Moldy cereals and legumes were mostly sold for animal feed. But sometimes it is used to process CFs during scarcity of cereals and legumes in some piloting Villages, that is why we collected for microbial and aflatoxin analysis. Overall, 40 moldy samples (each 2 kg) were collected across all the regions. Either cereals or legumes moldy samples were collected at each Villages GBs. However, similar types of cereal or legume were collected from the two selected Villages in each Woreda, so as to make one composite sample later at AAU to represent each District.

- 40 = 2 per District (one mixed sample of 2 kg collected in each Village) x 5 District x 4 Regions.

2) Sampling stage-2 (Pre-milling cereals and legumes):

The pre-milling cereals and legumes samples were made from cereals and legumes brought by mothers for bartering and stored in the GBs for CFs processing. We collected 2 kg of cereals and 2 kg of legumes based on the availability of each ingredient at each GBs. For example, if there were four containers of maize in a given GB, then ~500 grams of maize were collected in a pattern distributed across four sides of each entire container and then mixed into one 2 kg composite sample. Then the samples (cereals and legumes) were cleaned manually, soaked, dehulled, dried and then roasted (light roasting). After roasting, the samples were cooled at room temperature for some times. After cooling, the roasted legume samples were coarsely ground (split) into pieces using stone mill by the trained mothers. Then the inside part was separated manually from the hull and used as pre-milling ingredient and for CFs preparation. Thus, a total of 2 kg of each pre-milled cereal and of each of legume were collected as pre-milling samples in each GB across all the selected Villages within a maximum of one day. Overall, 132 pre-milling cereals and legumes "baseline" samples (each 2 kg) were collected across all the regions. The proportion of pre-milling cereals to legumes was a minimum of 1:1 in SNNP region and a maximum of 3:2 in Amhara region.

- 132= 48 Amhara region + 30 Tigray region + 30 Oromia region +24 SNNP region.

3) Sampling stage-3 (Post production CFs):

Post production CFs samples (CFs made from 70:30 proportions of cereals and legumes respectively) were CFs flour made by mixing the pre-milled cereals and legumes and grinded (milled) together according to the proportions of cereals to legumes in each region. The maximum proportions of cereals to legumes are 3:2 (Amhara region) and the minimum is 1:1 (SNNP region). The 70:30, cereals to legumes proportions are used to prepare CFs so as to improve the energy density and the protein quality of the CFs. This 70:30 proportions have been used in many countries (Ejigui *et al*., 2007).

Overall, 40 post production CFs samples were collected from the GB across all regions. This was the same type of samples mother took to their homes and put at the GBs for duration of one month. Here additional amounts of CFs (2 kg) was given to the mothers so as to collect the left over after one month to evaluate the handling practices at HHs. A maximum of 2 days was used in each piloting District to process and collect the post production CFs across all the regions.

- 40= 2 in each Woreda x 5 Woreda per region x 4 regions.

Note: The post production CFs samples were a "base line" samples to evaluate if HH and GB storage practices increase the levels of aflatoxin and food borne microbial agents due to poor handling and storage practices.

4) Sampling stage-4 (Post production CFs stored for one month both at HHs and GBs):

A total of 80 post production CFs samples stored for one month (each 2 kg); 40 from HHs and 40 from GBs was collected from both HHs and GBs after one-month storage time.

- 80= 4 per District (2 from HH and 2 from GB) x 5 Districts per region x 4 regions.

4.4.3 Sampling handling and shipment

Individual 2 kg of each sample (moldy, pre-milling, post production CFs, and CFs (HH) and CFs (GB) were collected in the field (HHs and GBs) in paper bags and kept in polyethylene bags with

300 gm desiccants (self-indicating silica gel) (Figure 4.2) and kept together in a plastic box with lids. This way the samples were transported from field to AAU (Figure 4.3 and Figure 4.4).

Figure 4.2 Self indicating silica gel

Figure 4.3 Sample handling with self-indicating silica gel at the field

Figure 4.4 Plastic box with lids for sample storage and transportation from site to AAU

The pre-milling and moldy samples collected were taken out from the polyethylene bags in Center for Food Science and Nutrition Research Laboratory and milled starting from the next day of arrival (Figure 4.5). Between batches of milling, the milling machine was cleaned properly using blower and rinsed by 70 % alcohol (ethanol) and then sanitized by grinding some portion of the next sample to avoid cross contamination.

Then for all the flour samples (moldy, pre-milling and CFs), a composite sample (2 kg) was prepared from aggregated samples of the two-selected villages from each District. Out of the 2-kg composite sample, 200 gm was shipped to centers for disease control and prevention (CDC), USA, for aflatoxin analysis (200 gm sample with 30 gm silica gel), 200 gm was kept at institute of biodiversity; the remaining 1.6 kg were used for analysis of microbiological safety parameters for *C. sakazakii*, coliforms and *E. coli*.

Figure 4.5 Sample milling and composing at Center for Food Science and Nutrition research laboratory, AAU

Overall, 146 (20 moldy, 66 pre-milling, 20 post-production CFs, and 40 one-month stored CFs) samples were collected in two rounds (106 samples first round and 40 samples second round) across all the regions (Table 4.2). The total number of samples collected in each region was different because of the differences in the availability of cereals and legumes perhaps the difference in the proportion of cereals and legumes (ratio of cereals: legumes) for CFs preparation.

Table 4.2 The total number of composite samples collected from the 20 piloting Districts

Region	District	Pre-milling ingredients		Moldy	Post-production	Post production one-month stored		Total
		Cereals	Legumes	Cereals/ legumes	CFs	CFs at HHs	CFs at GBs	
Amhara	Baso Liben	3	2	1	1	1	1	9
	Awabel	3	2	1	1	1	1	9
	Macakel	3	2	1	1	1	1	9
	Burie	3	1	1	1	1	1	8
	Sekela	3	2	1	1	1	1	9
	Total	**15**	**9**	**5**	**5**	**5**	**5**	**44**
Tigray	Endemekhoni	2	1	1	1	1	1	7
	Atsbi Womberta	2	1	1	1	1	1	7
	Kilte Awlalo	2	1	1	1	1	1	7
	Samre Saharti	2	1	1	1	1	1	7
	Adwa	2	1	1	1	1	1	7
	Total	**10**	**5**	**5**	**5**	**5**	**5**	**35**
Oromia	Midega Tole	2	1	1	1	1	1	7
	Merti	2	1	1	1	1	1	7
	Sire	2	1	1	1	1	1	7
	Kore	2	1	1	1	1	1	7
	Boset	2	1	1	1	1	1	7
	Total	**10**	**5**	**5**	**5**	**5**	**5**	**35**
SNNP	Geta	2	2	1	1	1	1	8
	Misrak Badawacho	1	1	1	1	1	1	6
	Aleta Chuko	1	1	1	1	1	1	6
	Shebedino	1	1	1	1	1	1	6
	Hula Tula	1	1	1	1	1	1	6
	Total	**6**	**6**	**5**	**5**	**5**	**5**	**32**
	All regions total	**41**	**25**	**20**	**20**	**20**	**20**	**146**

HH-household, GB-grain bank, CFs-complementary foods.

The basic information about the moldy, Pre-milling cereals and legumes and CFs samples collected across all the regions with their respective piloting Districts are depicted in Table 4.3, 4.4, 4.5, and 4.6.

Table 4.3 Basic information on the type of pre-milling ingredients, moldy samples and CFs collected in Amhara region

Region	District	The two rural Villages selected	Pre-milling ingredients	Ratio (cereals: legumes)	CFs formulation (cereals: legumes)	Moldy samples
Amhara	Baso Liben	Yelemlem & Limichm	Maize, wheat, teff & broad bean, pea	3:2	70:30	Teff
	Awabel	Zinda & Wejel Ankirak	Maize, wheat, teff & broad bean, chick pea	3:2	70:30	Maize
	Machakel	Kidamin Zuria & Yedefas	Maize, wheat, teff & broad bean, pea	3:2	70:30	Wheat
	Burie	Denbun and Fezel	Wheat, maize, teff & broad bean	3:1	70:30	Wheat
	Sekela	GishAbay & Kolel	Maize, wheat, teff & broad bean & pea	3:2	70:30	Broad bean

Table 4.4 Basic information on the types of pre-milling ingredients, moldy samples and CFs collected in Tigray region

Region	District	The two rural Villages selected	Pre-milling ingredients	Ratio (cereals: legumes)	CFs Formulation (cereals: legumes)	Moldy samples
Tigray	Endemekhoni	Embahasti & Shimta	Wheat, maize & pea	2:1	70:30	Wheat
	Atsbi Womberta	Felege Weyni & Gebrekidan	Wheat, maize & pea	2:1	70:30	Maize
	Kilte Awlalo	Debre-tsion & Negash	Wheat, teff & broad bean	2:1	70:30	Broad bean
	Samre Saharti	Amdi-Weyane & May-tekli	Wheat, maize & broad bean	2:1	70:30	Maize
	Adwa	Debre Genet & Weyenti	Wheat, teff & broad bean	2;1	70:30	Teff

Table 4.5 Basic information on the type of pre-milling ingredients, moldy samples and CFs collected in Oromia region

Region	District	The two rural Villages selected	Pre-milling ingredients	Ratio (cereals: legumes)	CFs Formulation (cereals: legumes)	Moldy samples
	Midega Tole	Berzala & Lencha	Sorghum, maize & pea	2:1	70:30	Sorghum
	Merti	Gora Silinko & Shemo Gado	Sorghum, wheat & broad bean	2:1	70:30	Sorghum
	Sire	Alelu Gasala & Ibsata Duga	Wheat, maize & haricot bean	2:1	70:30	Haricot bean
Oromia	Kore	Doda Deyyu & Bulchana Hulluko	Barely, maize & broad bean	2:1	70:30	Maize
	Boset	Dongorre Tiyo & Dongorre Furda	Maize, teff & chick pea	2:1	70:30	Maize

Table 4.6 Basic information on the type of pre-milling ingredients, moldy samples and CFs collected in SNNP region

Region	District	The two rural Villages selected	Pre-milling ingredients	Ratio (cereals: legumes)	CFs Formulation (cereals: legumes: Sugar)	Moldy samples
	Geta	S/Koreficha & Agata	Barley, wheat & broad bean, pea	2:2	70:20:10	Barley
	Misrak Badawacho	Anburse Anjulo & Kenchera	Maize & haricot bean	1:1	70:30:0	Maize
	Aleta Chuko	C/Lamala & Weyama	Maize & haricot bean	1:1	70:30:0	Maize
SNNP	Shebedino	M/Negash & M/ Qutela	Maize & haricot bean	1:1	70:30:0	Maize
	Hula Tula	Finchawa & Tulo	Maize & haricot bean	1:1	70:30:0	Maize

4.4.4 Determination of *C. sakazakii*

C. sakazakii was detected from the food samples based on the method ISO/TS 22964:2006 at Bless Agri Food, ISO 17025 accredited laboratory.

Method principle: The pre-enrichment medium was inoculated with the test portion and incubated at 37^{0}C for 24 hr and then inoculated to the selective enrichment medium and incubated at 44^{0}C for 24 hr. A Chromogenic agar was inoculated with the enrichment culture obtained from selective enrichment and incubated at 44^{0}C for 24 hr. Then typical colonies were selected from chromogenic agar, and isolates producing a yellow pigment on tryptone soya agar (TSA) were biochemically characterized.

Equipment used: Balance, incubator (25^{0}C, 37^{0}C, 44^{0}C), autoclave (for wet sterilization), pipettes (1ml, 2ml, 10ml), oven (for dry sterilization), water bath (capable of being maintained at 44^{0}C) and P^H meter.

Media and reagents used: Recognized analytical grade reagents were used. Distilled water that is free from substances that might inhibit the growth of microorganisms under the test condition was used. Besides, culture media was prepared by following the manufacturer's instruction carefully.

Culture media used: Buffered peptone water (BPW), modified lauryal sulphate tryptose broth (MLST)/ vancomycin medium, modified lauryal sulphate tryptose broth (MLST), vancomycin solution, enterobacter sakazakii isolation agar (ESIA) and tryptone soya agar (TSA).
Media and reagents used for biochemical characterization: Reagent for detection of oxidase, L-Ornithine decarboxylation medium, media for fermentation of carbohydrates and simmons citrate medium.

Procedure:

- ✓ Test portion of the sample, 10 gm was measured using conical flask and dissolved by 90 ml of buffered peptone water (pre-enrichment medium) to 1:9 dilutions.
- ✓ The dry sample was mixed gently to disperse in the liquid medium.
- ✓ **Pre-enrichment**: the inoculated pre-enrichment medium was incubated at 37^{0}C for 24 hr.
- ✓ **Selective enrichment**: 0.1 ml of obtained culture was transferred in to 10 ml MLST /0.1ml vancomycin medium using two tubes and incubated at 44^{0}C for 24 hr. Water bath was used to ensure that the maximum temperature (44^{0}C) is not exceeded.

Isolation of presumptive *C. sakazakii*:

- ✓ After incubation of the inoculated MLST/vancomycin medium, a loopful (ca.10 micro litters) was streaked on to the surface of the enterobacter sakazakii isolation agar plate and the plate was incubated at 44^0C for 24 hr.
- ✓ After incubation, the chromogenic plate for the presence of typical colonies of presumptive *C. sakazakii* was examined.
- ✓ **Note**: typical colonies were small to medium sized (1 mm to 3 mm) green to blue green colonies. Non-typical colonies were often slightly transparent and violet colored.

Confirmation:

a. Production of a yellow pigment:

- ✓ Selection of colonies: one to five of the typical colonies of presumptive *C. sakazakii* was selected and examined on the incubated chromogenic plate.
- ✓ Incubation: the selected colonies were streaked on to the surface of the TSA plate and the plate was incubated at 25^0C for 48 hr.
- ✓ After incubation, the TSA plate was examined for the presence of yellow pigmented colonies.

b. Biochemical confirmation:

One yellow pigmented colony from each TSA plate was selected for further biochemical characterization according to the following.

- ✓ **Oxidase**: a portion of each selected characteristic colony was taken using disposable inoculation needle. The taken portion was streaked on a filter paper moistened with oxidase reagent. The test portion was considered to be negative when the filter paper was not changed to mauve (pale purple), violet or deep blue within 10 seconds.
- ✓ **L-Ornithine decarboxylase**: the selected colonies were inoculated in to the L-Ornithine decarboxylation medium using a loop. Then the tubes were incubated at 30^0C for 24 hr. A violet color after incubation indicated a positive reaction while a yellow color indicated a negative reaction.

- ✓ **Fermentation of various sugars**: the selected colonies were inoculated in to carbohydrate fermentation medium (D-sorbitol, L-rhamnose, D-sucrose, D-melibiose) using a loop. Then the tubes were incubated at 30^{0}C for 24 hr. A yellow color after incubation indicates a positive reaction while a red color indicates a negative reaction.
- ✓ **Utilization of citrate**: the selected colonies were streaked on to the slant of simmons citrate medium. Then the tubes were incubated at 30^{0}C for 24 hr. The reaction was positive if the medium turns blue.

Interpretation of the results: The result was interpreted according to Table 4.7.

Table 4.7 Interpretation of *C. sakazakii* results

Confirmatory test	Positive or negative reaction	Percent of *C. sakazakii* strains showing reaction
Production of a yellow pigment	+	>99
Oxidase	-	>99
L-Ornithine decarboxylase	+	±90
Fermentation of D-sorbitol	-	±95
Fermentation of L-rhamnose	+	>99
Fermentation of D-sucrose	+	>99
Fermentation of D-melibiose	+	>99
Hydrolysis of citrate	+	>95

Expression of the results: After confirmation by confirmatory test procedure, of one or more of the presumptive *C. sakazakii* obtained in presumptive C. *sakazakii* test procedure, the presence or absence (+ or -) of *C. sakazakii* was reported in the test portion.

4.4.5 Determination coliforms and *E. coli*

Coliforms and *E. coli* were analyzed by using the conventional method called Most Probable Number (MPN) method according to FAO (1997).

Method principle: Graduated amount of food (diluted) sample were transferred to a series of fermentation tubes containing lauryl sulphite tryptose broth of proper strength, it was usual practice to inoculate to three fermentative tubes. The tubes were incubated at 35^0C for 24 and 48 hrs. The formation of gas in any of the tubes within 48 hr, regardless of the amount constitutes as positive for coliform and the absence of gas formation within this period considered as negative for coliform. The coliform was confirmed by using brilliant green lactose bile (BGLB) broth.

Equipment and materials used: Covered water bath, incubator, balance, blender and blender jar, sterile graduated pipets, sterile utensils for sample handling, dilution bottles made of borosilicate glass with stopper or polyethylene caps equipped with teflon liners, quebec colony counter (or equivalent with magnifying lens), long wave UV light, PH meter.

Materials and reagents used: Brilliant green lactose bile (BGLB) broth 2%, lauryl tryptose (LST) broth, EC broth, levine's eosin-methylene blue (L-EMB) agar, tryptone (tryptophane) broth, MR-VP broth, koser's citrate broth, plate count agar (PCA), butterfields phosphate-buffered dilution water, voges-proskauer (VP) reagents, methyl red indicator.

Presumptive test for coliform bacteria:

- ✓ Ten-gram (10 gm) sample was weighed with conical flask and 90 ml butterfield's phosphate buffered dilution water was added and mixed for 2 mints. Then three consecutive serial dilutions (1, 1, 1; 2, 2, 2; 3, 3, 3) were prepared.
- ✓ **First dilution**: 1 ml sample solution was taken and added to the first LST containing tubes that contained durham tube inside.
- ✓ **Second dilution**: 5 ml sample solution was taken from the original sample solution and 45 ml butterfield phosphate buffered dilution water was added on it to make 1:9 dilutions. Then 1 ml was taken from this solution and added to the second LST containing tubes.
- ✓ **Third dilution**: 5 ml was taken from the second dilution sample and 45 ml butterfield phosphate buffered dilution water was added on it to make 1:9 dilutions again. Then 1ml was taken from this solution and added to the third LST containing tubes.
- ✓ Then the three consecutive test tubes (three each) were incubated at 35^0C for 48 hr. The tubes were examined at 24 hr for gas production i.e. displacement of medium in

fermentation vial or effervescence when tubes were gently agitated. The negative tubes were re incubated for additional 24 hr and examined for gas production for the second time. Then a confirmed test was performed on all presumptive positive (gassing) tubes.

Confirmed test for coliforms:

- ✓ Each gassing LST tube was gently agitated and loopful of suspension was transferred to tube of BGLB broth. Hold LST tube at an angle and loop was inserted to avoid transfer of pellicle (if present).
- ✓ The BGLB tubes were incubated at 35^0C for 48 hr.
- ✓ Gas production was examined and recorded.
- ✓ Then MPN of coliforms based on proportion of confirmed gassing LST tubes for the 3 consecutive dilutions was calculated based on the MPN index (Annex B).

EC broth method for fecal coliforms and confirmed test for *E. coli*:

- ✓ Each gassing LST tube was gently agitated and loopful of each suspension was transferred to tube of EC broth.
- ✓ The EC tubes were incubated at 45.5^0C for 48 hr.
- ✓ Gas production was examined at 24 hr, if negative at 48 hr.
- ✓ Results of this test were used to calculate fecal coliform MPN.
- ✓ Then, loopful of suspension from each gassing tube was streaked to L-EMB agar and incubated at 35^0C for 24 hr.
- ✓ Plates were examined for suspicious *E. coli* colonies, i.e. dark centered and flat, with or without metallic sheen.
- ✓ Two suspicious colonies from each LEMB plate was transferred to PCA slants for morphological and biochemical tests.
- ✓ The PCA slants were incubated at 35^0C for 24 hr.
- ✓ Then one colony from every plate was used (picked) to examine the following biochemical activities.

- ✓ **Voges-Proskauer (VP) reactive compounds**: the suspected *E. coli* colony from the PCA slants were inoculated to tube of MR-VP broth and incubated at 35^0C for 48 hr. 1 mL was transferred to 13 X 100 mm tube and 0.6 ml alpha-naphthol solution and 0.2 ml 40% KOH was added on it and the mixture was shaken for some minutes. Then a few crystals of creatine were added and shaken and let it stands for 2 hr. The test was positive if eosin pink color develops.
- ✓ **Methyl red reactive compounds**: after VP test, the inoculate MR-VP tube broth was incubated for additional 48 hr at 35^0C. Then 5 drops methyl red solution was added to each tube. Distinct red color was positive test while yellow was negative reaction.
- ✓ **Citrate**: inoculate to tube of Koser's citrate broth and incubated at 35^0C for 96 hr. Development of distinct turbidity was positive reaction.
- ✓ **Gas from lactose**: inoculate tube of LST broth and incubated at 35^0C for 48 hr. Displacement of medium from inner vial or effervescence after gentle agitation was positive reaction.
- ✓ **Interpretation**: all cultures that ferment lactose with production of gas within 48 hr at 35^0C, appear as Gram-negative non-spore forming rods or cocci, and give IMViC patterns ++-- (biotype 1) or -+-- (biotype 2) was considered to be *E. coli*. Then MPN of *E. coli* was calculated based on proportion of EC tubes in 3 successive dilutions that contain *E. coli* (Annex B).

4.4.6 Data analysis

Data were entered into SPSS 20 statistical software. The data were analyzed, presented and reported using descriptive statistics methods. Important indicators were computed for each region.

4.5 Results and Discussion

4.5.1 Microbiological quality of moldy cereals and legumes

Moldy cereals and legumes are used to prepare CFs during scarcity of CFs ingredients in some parts of the Districts. That is why we used to evaluate the microbiological quality of moldy cereals and legumes. Twenty moldy cereals and/or legumes samples were collected from all the regions and the presence of *C. sakazakii*, coliforms and *E. coli* were detected and the results are depicted in (Table 4.8). The overall incidence of *C. sakazakii*, coliforms and *E. coli* were 15%, 60% and 15%, respectively.

C. sakazakii was detected in 15% (3/20) of the moldy samples collected from the entire regions. Coliforms were detected for the majority of the samples collected across the entire regions, 60% of the samples were contaminated by coliforms at a level of >1100 CFU/g. Further, 15% (3/20) of the moldy samples collected from the entire region were contaminated by *E. coli* at a level of 23 CFU/g. The contamination of the moldy samples by *C. sakazakii*, coliforms and *E. coli* reflects the poor hygienic practices undergoing at the GBs.

Table 4.8 The microbiological quality of moldy cereals and legumes sorted out during CFs production

Region	District	Moldy cereals/legumes	*C. sakazakii* Per 10 g	Presumptive coliforms count (CFU/g)	*E. coli* (CFU/g)
Amhara	Baso Liben	Teff	-	75	<3
	Awabel	Maize	-	<3	<3
	Machakel	Wheat	-	<3	<3
	Burie	Wheat	+	9	4
	Sekela	Broad bean	-	23	23
	Mean		1/5 (20%)	3/5 (60%)	2/5 (40%)
Tigray	Endemekhoni	Wheat	-	<3	<3
	Atsbi Womberta	Maize	-	>1,100	4
	Kilte Awlalo	Broad bean	-	9	<3
	Samre Saharti	Maize	-	>1,100	<3
	Adwa	Teff	-	460	<3
	Mean		0/5 (0%)	4/5 (80%)	1/5 (20%)
Oromia	Midega Tole	Sorghum	-	<3	<3
	Merti	Sorghum	-	<3	<3
	Sire	Haricot bean	-	43	<3
	Kore	Maize	+	<3	<3
	Boset	Maize	-	93	<3
	Mean		1/5 (20%)	2/5 (40%)	0/5 (0%)
SNNP	Geta	Barely	-	4	<3
	MisrakBadwacho	Maize	-	<3	<3
	Aleta Chuko	Maize	+	<3	<3
	Shebedino	Maize	-	210	<3
	Hula Tula	Maize	-	1,100	<3
	Mean		1/5 (20%)	3/5 (60%)	0/5 (0%)
All Regions mean of incidence			3/20 (15%)	12/20 (60%)	3/20 (15%)

4.5.2 Microbiological quality of pre-milling ingredients

The presence of *C. sakazakii*, coliforms and *E. coli* were detected from the pre-milling cereals and legumes used for the production of CFs in Amhara region (Table 4.9). Overall, 17%, 36.8%, and 0% of the samples were found to be positive in *C. sakazakii*, coliforms and *E. coli,* respectively. The positive strains of *C. sakazakii* formed yellow colonies on TSA plate after 48 hr of incubation at 25^0C (Figure 4.6). Although these pre-milling ingredients are exposed to heat treatments (roasting), but *C. sakazakii* was still detected. This might be due to post-processing contamination at the GBs.

Table 4.9 The microbiological quality of pre-milling cereals and legumes used for CFs production in Amhara region

Region	District	Pre-milling cereals/legumes	*C. sakazakii* Per 10 g	Presumptive coliforms count (CFU/g)	*E. coli* (CFU/g)
	Baso Liben	Maize	-	> 1,100	<3
		Teff	-	<3	<3
		Wheat	-	<3	<3
		Broad bean	-	<3	<3
		Pea	-	<3	<3
		Mean	(0/5) 0%	1/5 (20%)	0/5 (0%)
	Awabel	Maize	-	<3	<3
		Teff	-	23	<3
		Wheat	+	<3	<3
		Broad bean	-	<3	<3
		Chick pea	+	43	<3
		Mean	2/5 (40%)	2/5 (40%)	0/5 (0%)
Amhara	Machakel	Maize	-	460	<3
		Teff	-	<3	<3
		Wheat	-	240	<3
		Broad bean	-	<3	<3
		Pea	-	<3	<3
		Mean	0/5 (0%)	2/5 (40%)	0/5 (0%)
	Burie	Maize	+	4	<3
		Teff	-	<3	<3
		Wheat	-	<3	<3
		Broad bean	+	<3	<3
		Mean	2/4 (50%)	1/4 (25%)	0/5 (0%)
	Sekela	Maize	-	<3	<3
		Teff	-	240	<3
		Wheat	-	<3	<3
		Broad bean	-	<3	<3
		Pea	-	<3	<3
		Mean	0/5 (0%)	1/5 (20%)	0/5 (0%)
Mean of incidence of the Region			4/24 (17%)	7/24 (36.8%)	0/24 (0%)

Presumptive coliforms count was detected in 36.8% (7/24) of the pre-milling ingredients at a level of 1,100 CFU/g. However, all the pre-milling ingredients collected from Amhara region were free from *E. coli* contamination. *E. coli* is an indicator organism that shows fecal contamination. Hence, from these results it can be concluded that mothers or HEWs working at the GBs are undergoing good hygienic practices (GHPs), particularly, washing hands with soap after defection.

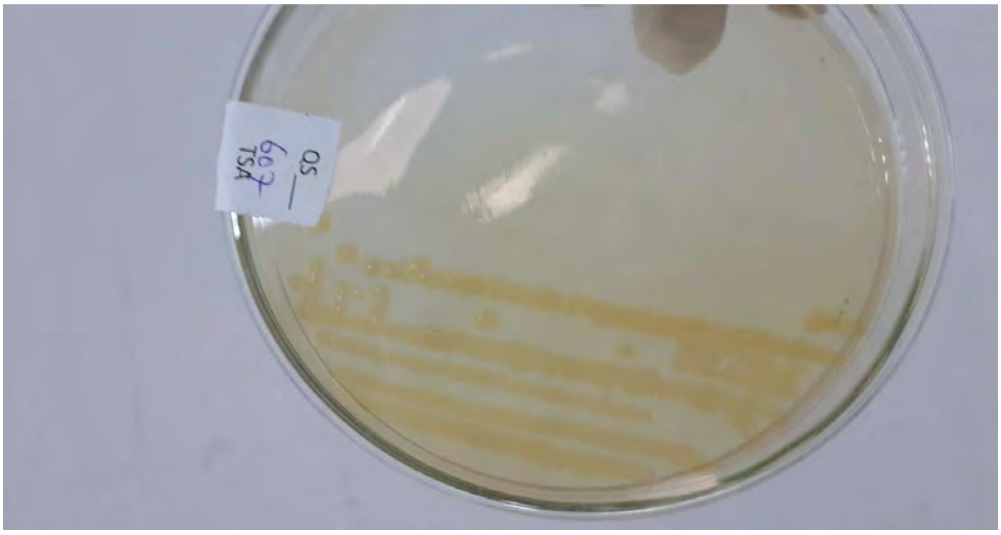

Figure 4.6 The growth of positive strains of *C. sakazakii* on TSA plate (yellow colonies)

Similarly, the microbiological quality of the pre-milling cereals and legumes used for CFs production in Tigray region is depicted in (Table 4.10). Overall, 20%, 6.7%, and 0% of the samples were found to be positive for *C. sakazakii*, coliforms and *E. coli*, respectively. *C. sakazakii* was detected in 20% (3/15) of the samples collected in the entire region. This might be due to poor sanitation practices at the GBs. However, this result is consistent with a previous study in Jordan by Shaker *et al*. (2007), who reported that *C. sakazakii* was detected from 33.3% of semolina (crushed wheat) samples. However, only 6.7% (1/15) of the samples were contaminated by coliforms count at a level of 4 CFU/g and all of the samples were free from *E. coli* contamination. From this result, it can be concluded that mothers/caregivers and/or HEWs practicing GHPs at the GBs.

Table 4.10 The microbiological quality of pre-milling cereals and legumes used for CFs production in Tigray region

Region	District	Pre-milling cereals/legumes	*C. sakazakii* Per 10 g	Presumptive coliforms count (CFU/g)	*E. coli* (CFU/g)
Tigray	Endemekhoni	Wheat	-	<3	<3
		Maize	-	<3	<3
		Pea	-	<3	<3
	Mean		0/3 (0%)	0/3 (0%)	0/3 (0%)
	Atsbi Womberta	Maize	-	<3	<3
		Wheat	-	<3	<3
		Pea	-	<3	<3
	Mean		0/3 (0%)	0/3 (0%)	0/3 (0%)
	Kilte Awlalo	Wheat	-	<3	<3
		Teff	+	<3	<3
		Broad bean	-	<3	<3
	Mean		1/3 (33%)	0/3 (0%)	0/3 (0%)
	Samre Saharti	Maize	-	<3	<3
		Wheat	-	4	<3
		Broad bean	+	<3	<3
	Mean		1/3 (33%)	1/3 (33%)	0/3 (0%)
	Adwa	Teff	-	<3	<3
		Wheat	+	<3	<3
		Broad bean	-	<3	<3
	Mean		1/3 (33%)	0/3 (0%)	0/3 (0%)
Mean of incidence of the Region			3/15 (20%)	1/15 (6.7%)	0/15 (0%)

Pre-milling ingredients collected from Oromia region were analysed for the presence of *C. sakazakii*, coliforms and *E. coli* (Table 4.11). Overall, 13.3%, 6.7%, and 0% of the samples were found to be positive for *C. sakazakii*, coliforms and *E. coli*, respectively. *C. sakazakii* was detected in 13.3% (2/15) of the samples collected in the entire region. Particularly wheat and teff samples were contaminated. This result is in agreement with a previous study in China by Lou *et al*. (2014) who reported *C. sakazakii* from wheat flours and also reported that wheat flour is one likely reservoir and/or transmission route for *C. sakazakii*. However, only 6.7% (1/15) of the sample was contaminated by Presumptive coliforms count at a level of 43 CFU/g and all the samples were free from *E. coli* contamination. The possible explanation for this is again GHPs undergoing at HHs and at GBs.

Table 4.11 The microbiological quality of pre-milling cereals and legumes used for CFs production in Oromia region

Region	District	Pre-milling Cereals/Legumes	*C. sakazakii* Per 10 g	Presumptive coliforms count (CFU/g)	*E. coli* (CFU/g)
Oromia	Midega Tole	Maize	-	<3	<3
		Sorghum	-	<3	<3
		Pea	-	43	<3
		Mean	0/3 (0%)	1/3 (33%)	0/3 (0%)
	Merti	Sorghum	-	<3	<3
		Wheat	+	<3	<3
		Broad bean	-	<3	<3
		Mean	1/3 (33%)	0/3 (0%)	0/3 (0%)
	Sire	Maize	-	<3	<3
		Wheat	-	<3	<3
		Haricot bean	-	<3	<3
		Mean	0/3 (0%)	0/3 (0%)	0/3 (0%)
	Kore	Barely	-	<3	<3
		Maize	-	<3	<3
		Broad bean	-	<3	<3
		Mean	0/3 (0%)	0/3 (0%)	0/3 (0%)
	Boset	Maize	-	<3	<3
		Teff	+	<3	<3
		Chick pea	-	<3	<3
		Mean	1/3 (33%)	0/3 (0%)	0/3 (0%)
Mean of incidence in the Region			2/15 (13.3%)	1/15 (6.7%)	0/15 (0%)

Similarly, pre-milling ingredients collected from SNNP region were analysed for the presence of *C. sakazakii*, coliforms and *E. coli* and the results are depicted in (Table 4.12). The overall incidence of *C. sakazakii*, coliforms and *E. coli* were found to be 8.3%, 0%, and 0%, respectively. Only 8.3% (1/12) of the sample was positive for *C. sakazakii*. However, all the samples were free from coliforms count and *E. coli* contamination. This result could reflect the good hygienic condition of the pre-milling cereals and legumes processing environments. Further, it can also indicate the good personal hygiene of mothers and/or HEWs during pre-milling ingredients preparation. An important way to prevent food from microbial contamination is to maintain good standards of personal hygiene and cleanliness (Omalu *et al*., 2013; Gomes *et al*., 2014).

Table 4.12 The microbiological quality of pre-milling cereals and legumes used for CFs production in SNNP region

Region	District	Pre-milling Cereals/Legumes	*C. Sakazakii* Per 10 g	Presumptive coliforms count (CFU/g)	*E. coli* (CFU/g)
SNNP	Geta	Barely	-	<3	<3
		Wheat	-	<3	<3
		Broad bean	+	<3	<3
		Pea	-	<3	<3
	Mean		1/4 (25%)	0/4 (0%)	0/4 (0%)
	Misrak Badwacho	Maize	-	<3	<3
		Haricot bean	-	<3	<3
	Mean		0/2 (0%)	0/2 (0%)	0/2 (0%)
	Aleta Chucko	Maize	-	<3	<3
		Haricot bean	-	<3	<3
	Mean		0/2 (0%)	0/2 (0%)	0/2 (0%)
	Shebedino	Maize	-	<3	<3
		Haricot bean	-	<3	<3
	Mean		0/2 (0%)	0/2 (0%)	0/2 (0%)
	Hula Tula	Maize	-	<3	<3
		Haricot bean	-	<3	<3
	Mean		0/2 (0%)	0/2 (0%)	0/2 (0%)
Mean of incidence in the Region			1/12 (8.3%)	0/12 (0%)	0/12 (0%)

Overall, Tigray region was the most prevalent (20% (3/15)) while SNNP region was the least 8.3% (1/12)) in terms of *C. sakazakii* contamination. In terms of coliforms contamination, Amhara region was the highest (36.8% (7/24)) while SNNP region was the least (0% (0/12)). However, all the pre-milling ingredients collected across the entire regions were free from *E. coli* contamination.

4.5.3 Microbiological quality of post production CFs

The post production CFs collected from Amhara, Tigray, Oromia, and SNNP regions were determined for the presence of *C. sakazakii*, coliforms and *E. coli* (Table 4.13). Overall, 5%, 65%, and 5% of the samples were found to be positive in *C. sakazakii*, coliforms and *E. coli*, respectively.

The percentage incidence of *C. sakazakii* positive sample is low (5% (1/20)), although it was greater than the acceptable hygiene levels (not detected in CFs). However, our results are consistent with a previous study by Kim *et al*. (2011) who reported the presence of *C. sakazakii* from cereal based follow up formulas. Another study by Gicova *et al*. (2013) reported *C. sakazakii* positive samples from cereal based powdered infant foods. A study by Yao *et al*. (2016) also reported 12% *C. sakazakii* positive sample in cereal based indigenous infant flours collected from public health care centres in Abidjan Cote d'Ivoire. Another study by Chap *et al*. (2009) also reported that *C. sakazakii* was isolated from 3% and 12% of follow up formulas and infant foods respectively. But, a study by Lou *et al*. (2014) in China reported more positive *C. sakazakii* samples (60% (3/5)) in wheat based CFs for infants.

Coliforms were detected from 65% (13/20) of the post production CFs collected from the entire regions at a level of 460 CFU/g (Table 4.13). However, only 5% (1/20) of the CFs collected from the entire regions were contaminated by *E. coli* at a level of 4 CFU /g. Although some of our samples are contaminated by coliforms and *E. coli*, the results are consistent with a study carried out by Islam *et al*. (2012) who reported that around 40% of the CFs given to the children was contaminated with a high number of total coliform bacteria ($\geq$100 CFU/g of food) and *E. coli*, which is indicative of direct faecal pollution. Another study by Adebayo-Tayo *et al*. (2012) reported the detection of coliform count (1.0 - 2.0 x 10^3 CFU/g) from cereal based baby food samples sold in Nigeria. A study by Kim *et al*. (2011) also reported the presence of coliforms from cereal based follow up formulas consumed by infants and babies in Korea. Another study by Kayalto *et al*. (2013) in Chad also reported <100 CFU/g and <10 CFU/g fecal coliforms and *E. coli* respectively in locally prepared infant flours.

Table 4.13 The microbiological quality of post production CFs collected across all the regions

Region	District	Post production CFs	*C. sakazakii* Per 10 g	Presumptive coliforms count (CFU/g)	*E. coli* (CFU/g)
Amhara	Baso Liben	M/W/T/BB/P	-	<3	<3
	Awabel	M/W/T/BB/CP	-	43	<3
	Machakel	M/W/T/BB/P	+	460	<3
	Burie	M/W/T/BB	-	<3	<3
	Sekela	M/W/T/BB/P	-	4	<3
	Mean		1/5 (20%)	3/5 (60%)	0/5 (0%)
Tigray	Endemekhoni	W/M/P	-	<3	<3
	Atsbi Womberta	W/M/P	-	<3	<3
	Kilte Awlalo	W/T/BB	-	20	4
	Samre Saharti	W/M/BB	-	<3	<3
	Adwa	W/T/BB	-	23	<3
	Mean		0/5 (0%)	2/5 (40%)	1/5 (20%)
Oromia	Midega Tole	S/M/P	-	240	<3
	Merti	S/W/BB	-	460	<3
	Sire	W/M/HB	-	<3	<3
	Kore	B/M/BB	-	9	<3
	Boset	M/T/CP	-	23	<3
	Mean		0/5 (0%)	4/5 (80%)	0/5 (0%)
SNNP	Geta	B/W/BB/P	-	<3	<3
	Misrak Badwacho	M/HB	-	4	<3
	Aleta Chuko	M/HB	-	23	<3
	Shebedino	M/HB	-	93	<3
	Hula Tula	M/HB	-	43	<3
	Mean		0/5 (0%)	4/5 (80%)	0/5 (0%)
All Regions mean of incidence			1/20 (5%)	13/20 (65%)	1/20 (5%)

Abbreviations: M - Maize, W - Wheat, T - Teff, BB - Broad bean, P - Pea, CP - Chick pea, S - Sorghum, HB - Haricot bean, and B - Barley.

4.5.4 Microbiological quality of post production one month stored CFs

The microbiological quality, (in terms of *C. sakazakii*, coliforms and *E. coli)* of the post production CFs stored for one month at HHs (CFs (HHs)) collected from Amhara, Tigray, Oromia, and SNNP regions are depicted in (Table 4.14). Overall, 5%, 80%, and 15% of the samples were found to be positive for *C. sakazakii*, coliforms and *E. coli*, respectively.

Table 4.14 The microbiological quality of CFs (HH) collected across all the regions

Region	District	CFs (HH)	*C. sakazakii* Per 10 g	Presumptive coliforms count (CFU/g)	*E. coli* (CFU/g)
Amhara	Baso Liben	M/W/T/BB/P	-	23	9
	Awabel	M/W/T/BB/CP	-	23	<3
	Machakel	M/W/T/BB/P	+	23	<3
	Burie	M/W/T/BB	-	<3	<3
	Sekela	M/W/T/BB/P	-	23	<3
	Mean		1/5 (20%)	4/5 (80%)	1/5 (20%)
Tigray	Endemekhoni	W/M/P	-	4	<3
	Atsbi Womberta	W/M/P	-	<3	<3
	Kilte Awlalo	W/T/BB	-	1,100	150
	Samre Saharti	W/M/BB	-	43	<3
	Adwa	W/T/BB	-	23	<3
	Mean		0/5 (0%)	4/5 (80%)	1/5 (20%)
Oromia	Midega Tole	S/M/CP	-	4	<3
	Merti	S/W/BB	-	9	<3
	Sire	W/M/HB	-	9	<3
	Kore	B/M/BB	-	4	<3
	Boset	M/T/CP	-	23	<3
	Mean		0/5 (0%)	5/5 (100%)	0/5 (0%)
SNNP	Geta	B/W/BB/P	-	43	43
	Misrak	M/HB	-	240	<3
	Aleta Chuko	M/HB	-	240	<3
	Shebedino	M/HB	-	<3	<3
	Hula Tula	M/HB	-	<3	<3
	Mean		0/5 (0%)	3/5 (60%)	1/5 (20%)
All Regions mean of incidence			1/20 (5%)	16/20 (80%)	3/20 (15%)

Abbreviations: M - Maize, W - Wheat, T - Teff, BB - Broad bean, P - Pea, CP - Chick pea, S - Sorghum, HB - Haricot bean, and B - Barley.

The percentage incidence of *C. sakazakii* positive sample is low (5% (1/20)), although it was above the acceptable hygiene levels (not detected in CFs). Coliforms were detected from 80% (16/20) of the CFs (HH) samples collected from the entire regions at a level of 1100 CFU/g. However, only 15% (3/20) of the samples collected from the entire regions were contaminated by *E. coli* at a level of 150 CFU/g. The positive strains of presumptive *E. coli* formed metallic sheen and black centered colonies on L-EMB agar incubated at 35^{0}C for 24 hr (Figure 4.7). The presumptive *E. coli* colony formed red color by adding methyl red solution on MR-VP tube broth incubated at 35^{0}C for 48 hr (Figure 4.8).

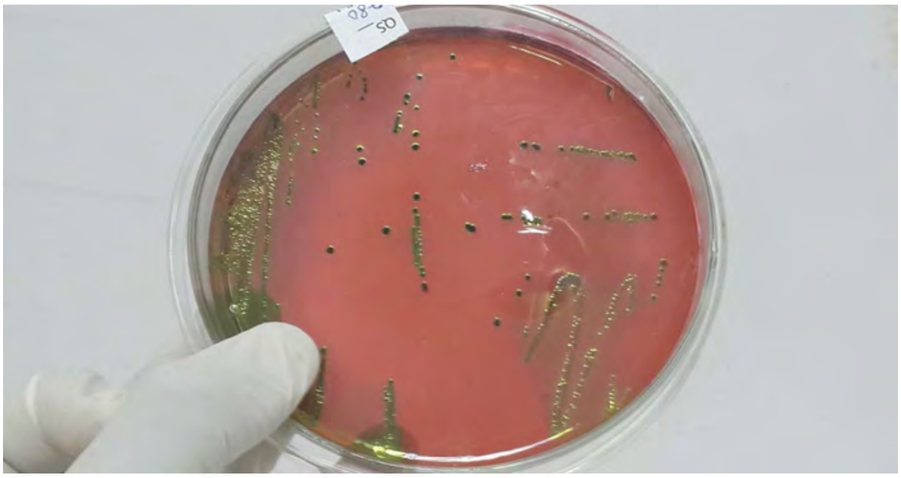

Figure 4.7 Typical presumptive *E. coli* colony growth on L-EMB agar (colonies with metallic sheen and black centered)

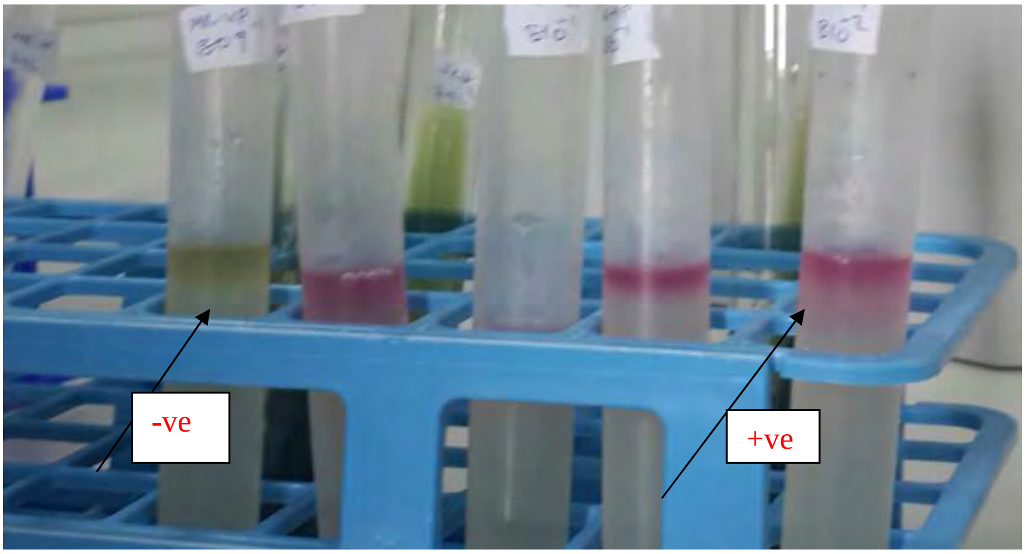

Figure 4.8 Biochemical confirmation of presumptive *E. coli* colony using methyl red test

Coliforms and *E. coli* numbers in the CFs (HHs) samples reported here are high. However, our results are consistent with a previous study by Islam *et al*. (2012) who reported that around 40% of the CFs given to the children was contaminated with a high number of coliform bacteria (>100 CFU/g of food) and *E. coli*, which is indicative of direct faecal pollution. Another study by Kung'u *et al*. (2009) also reported high incidence of coliforms in cooked porridge made from locally prepared CFs indicating the presence of higher amounts of coliforms count in the CFs flour.

The contamination of CFs samples by coliforms and *E. coli* reflects the poor personal hygiene of the mothers/caregivers and the poor hygienic practices undergoing at HHs. Poor socio-economic status, use of communal latrines, use of contaminated water for washing, low level of education or lack of appropriate knowledge and hygienic practices might be related to higher level of contamination (Islam *et al*., 2012).

The microbiological quality, (in terms of *C. sakazakii*, coliforms and *E. coli*) of the post production CFs stored for one month at GBs (CFs (GBs)) collected from Amhara, Tigray, Oromia, and SNNP regions are depicted in (Table 4.15). Overall, 10%, 80%, and 10% of the samples were found to be positive for *C. sakazakii*, coliforms and *E. coli*, respectively. *C. sakazakii* was detected from 10% (2/20) of the CFs (GBs) samples collected from the entire regions. The percentage incidence of *C. sakazakii* was relatively low, but it was above the acceptable hygiene levels (Not detected in CFs). However, our result is lower than a previous study by Lou *et al*. (2014) in China who reported (60% (3/5)) incidence of *C. sakazakii* in wheat based CFs for infants.

Coliforms were detected from the majority of the samples (80% (16/20)) collected from the entire regions at a level of 150 CFU/g. However, only 10% (2/20) of the CFs samples collected from the entire region was contaminated by *E. coli* at a level of 9 CFU/g. Generally, the contamination of CFs samples by coliforms and *E. coli* reflect the poor hygienic practices undergoing at GBs. Cross contamination from the storage environments might be the reason for the presence of these microbial contaminants in the CFs. Hence, hygienic practices at the GBs needs to be improved.

Table 4.15 The microbiological quality of CFs (GB) collected across all the regions

Region	District	CFs (GB)	***E. sakazakii*** **Per 10 g**	**Presumptive coliforms count (CFU/g)**	***E. coli*** **(CFU/g)**
Amhara	Baso Liben	M/W/T/BB/P	-	43	<3
	Awabel	M/W/T/BB/CP	-	23	4
	Machakel	M/W/T/BB/P	+	23	9
	Burie	M/W/T/BB	-	4	<3
	Sekela	M/W/T/BB/P	-	23	<3
	Mean		1/5 (20%)	5/5 (100%)	2/5 (40%)
Tigray	Endemekhoni	W/M/P	-	<3	<3
	Atsbi Womberta	W/M/P	-	4	<3
	Kilte Awlalo	W/T/BB	-	43	<3
	Samre Saharti	W/M/BB	-	<3	<3
	Adwa	W/T/BB	-	9	<3
	Mean		0/5 (0%)	3/5 (60%)	0/5 (0%)
Oromia	Midega Tole	S/M/CP	-	150	<3
	Merti	S/W/BB	-	4	<3
	Sire	W/M/HB	-	23	<3
	Kore	B/M/BB	-	<3	<3
	Boset	M/T/CP	-	43	<3
	Mean		0/5 (0%)	4/5 (80%)	0/5 (0%)
SNNP	Geta	B/W/BB/P	-	<3	<3
	Misrak	M/HB	+	23	<3
	Aleta Chuko	M/HB	-	23	<3
	Shebedino	M/HB	-	93	<3
	Hula Tula	M/HB	-	23	<3
	Mean		1/5 (20%)	4/5 (80%)	0/5 (0%)
All Regions mean of incidence			2/20 (10%)	16/20 (80%)	2/20 (10%)

Abbreviations*:* M - Maize, W - Wheat, T - Teff, BB - Broad bean, P - Pea, CP - Chick pea, S - Sorghum, HB - Haricot bean, and B - Barley.

4.5.5 The microbial load of post production CFs versus one month stored CFs

The percentage incidences of *C. sakazakii* from post production CFs, CFs (HH) and CFs (GB) were 5%, 5%, and 10%, respectively (Figure 4.9). The highest percentage *C. sakazakii* incidence at the GBs might be due to the poor storage practices and cross contaminations.

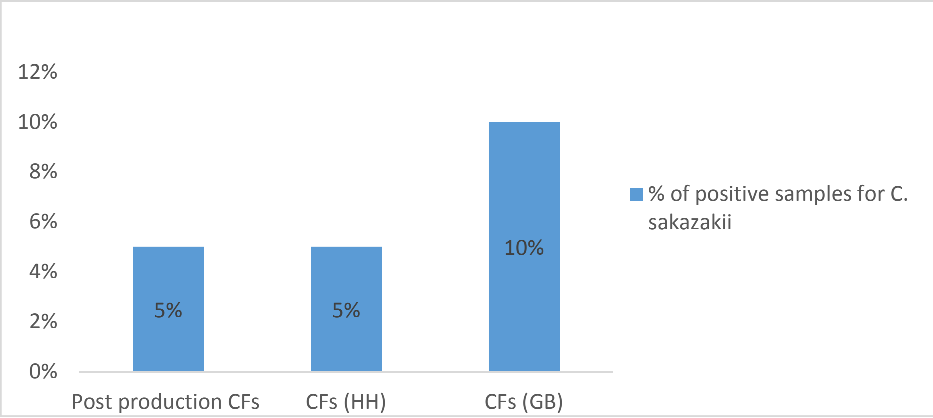

Figure 4.9 Comparison of positive samples for *C. sakazakii* in CFs from post production to following one-month storage in HHs and GBs across all regions

The percentages of incidence of coliforms and *E. coli* were also increased in CFs from post production to following one-month storage in HHs and GBs across all regions (Figure 4.10 and 4.11). The percentage of incidences of coliforms and *E. coli* were found to be 65%, 80%, & 80% and 5%, 15% & 10%, respectively. As can be shown from Figure 4.11, the incidences of *E. coli* in HHs are relatively greater than GBs which reflects the poor hygienic practices at HHs.

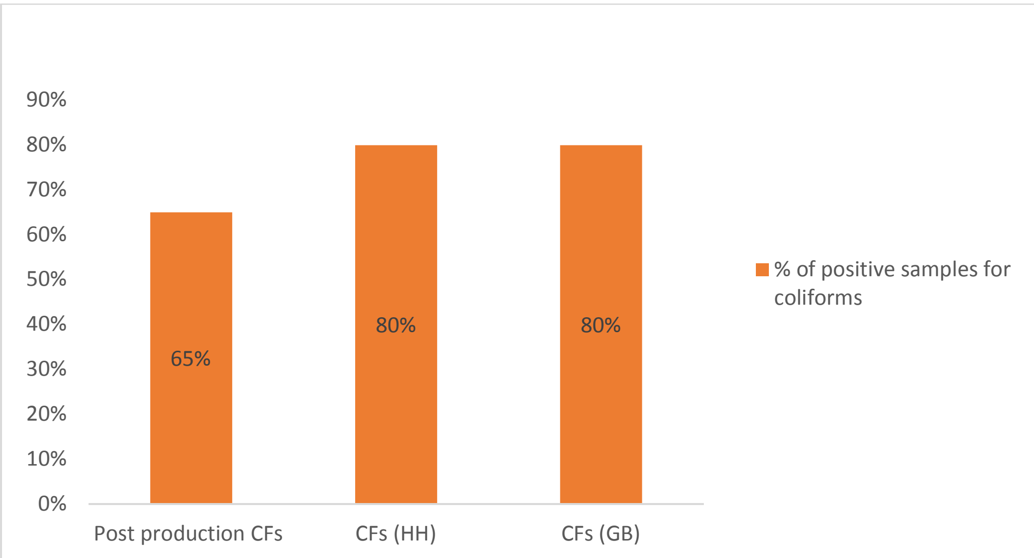

Figure 4.10 Comparison of positive samples for coliforms in CFs from post production to following one-month storage in HHs and GBs across all regions

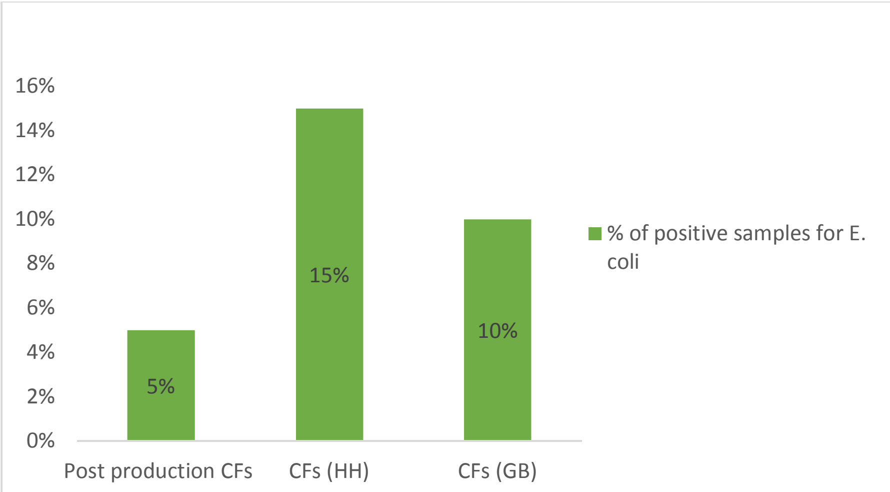

Figure 4.11 Comparison of positive samples for *E. coli* in CFs from post production to following one-month storage in HHs and GBs across all regions

4.6 Conclusion

This study evaluated the microbial safety of 146 samples. Of which, almost half of the samples were positive in coliforms but very few samples were positive in *C. sakazakii* and *E. coli*. The percentage incidences of both *C. sakazakii*, coliforms and *E. coli* were higher in one month stored CFs both at households and grain banks, which implies poor hygienic practices or cross-contamination from the processing equipment and storage environment. Thus, the presence of these food borne microbial agents in CFs might have a potential heath impacts to the young children in the study areas. Therefore, the hygienic practices both at households and grain banks needs to be improved to reduce and/or prevent the CFs from microbial contamination.

CHAPTER 5. ESTIMATION OF TOTAL AFLATOXINS IN COMPLEMENTARY FOODS PRODUCED AT COMMUNITY LEVELS USING LOCALLY AVAILABLE INGREDIENTS IN ETHIOPIA

5.1 Abstract

Aflatoxins are a group of toxic secondary metabolites produced by filamentous fungi as part of their secondary metabolism. They may be present in raw and processed food, including cereals and cereal-based complementary foods (CFs). Hence consumption of these commodities may cause a potential health risk to consumers, particularly young children. This study was conducted to determine the occurrence and levels of total aflatoxins in purposively removed moldy cereals and legumes, pre-milling cereals and legumes, post-production CFs, and one-month post-production CFs stored at grain banks (GBs) and in households (HHs). A total of 146 samples collected from 20 Districts from Amhara, Tigray, Oromia and Southern Nations Nationalities and Peoples (SNNP) regions were analyzed for levels of total aflatoxins using enzyme linked immunosorbent assay (ELISA). Aflatoxins were detected in 19 out of 20 moldy cereals and legume samples with mean range of 2.3 - 88 µg/kg. In pre-milling cereals and legumes, aflatoxins were detected in 62 out of 66 samples with mean range of 0.3 - 9.9 µg/kg. Aflatoxins were also detected in 19 out of 20 post-production CFs samples, and in all of the one-month post-production CFs stored at HHs and GBs, with mean range of 0.5 - 8.0 µg/kg, 3.6 - 11.3 µg/kg and 0.2 - 12.4 µg/kg, respectively. Overall, five out of 146 samples exceeded the acceptable limit (10 µg/kg), all of which were either purposively removed moldy samples or post one-month of storage. Although most aflatoxin levels were considered safe for consumption, more effort should be implemented to reduce contamination, particularly as these CFs are intended for consumption by young children. Further studies need to be conducted to assess exposure to aflatoxins and association between aflatoxin exposure and child stunting in Ethiopia.

Keywords: Aflatoxin; Complementary food; Cereals; Legumes; Grain banks; Households.

5.2 Introduction

The nutritional status of Ethiopian children is poor, including stunting among 40% of children less than 5 years of age, which is the highest in Sub Saharan Africa (CSA, 2014). Such a high prevalence of undernutrition reaches its peak during the period when complementary foods (CFs) are typically introduced, indicating that inappropriate introduction and patterns of complementary feeding may contribute to the problem. UNICEF-Ethiopia and partners, recognizing this problem, developed a project for the local production of CFs that would improve the nutritional intake, and thus status of children 6-23 months in rural settings of Amhara, Tigray, Oromia and Southern Nations Nationalities and Peoples (SNNP) regions.

The project organizes the community to initiate and implement a centralized production and distribution of CFs through a bartering system. The project provides limited equipment (storage bins, roasting pans, bags, balance and sealer) and most of the raw products (cereals/legumes) are contributed by the community, depending on their availability. These are stored in a grain bank (GB) (a space provided by the community used to prepare and store the CFs). Women are identified and trained to process the CFs by following a standard formulation developed by the Center for Food Science and Nutrition, AAU. The bartering system invites mothers to bring 2 kg of raw materials and received 3 kg of prepares CFs in return.

The project has been well integrated and accepted by the communities involved in the pilot. However, there are concerns by which the CFs and its ingredients might have the tendency to be contaminated by mold and aflatoxin. Since previous findings reported high aflatoxin content in certain cereals (maize, wheat, teff, sorghum, finger millet and barley) in Ethiopia that are potentially used for the production of CFs (Ayalew *et al*., 2006; Ayalew, 2010; Chala *et al*., 2014).

Aflatoxins are toxic secondary metabolites produced by filamentous fungi (Calvo *et al*., 2002). Aflatoxins contaminate many food crops, but cereals and groundnuts being the most susceptible (Reddy *et al*., 2010; Wild & Gong, 2010; Yard *et al*., 2013). Contamination of cereals and cereal based products (CFs) by mold and aflatoxins results in quality and nutritional losses and represents a substantial threat to the food chain (Magan & Aldred, 2007).

The risks of mold and aflatoxin contaminations occurs in the field, during storage, transportation, during processing and after processing. It appears to be encouraged by factors such as drought, high humidity, insect infestation and sub-optimal harvesting, drying and crop storage practices. However, it occurs mostly during storage, that are mostly favoured by the moisture content of the substrate and the relative humidity of the surroundings (Bennett & Klich, 2003). In addition to the aforementioned factors, a lack of consistently enforced aflatoxin regulatory limits, cause people in developing countries to be more adversely affected by aflatoxin exposure (Wild & Gong, 2010; Williams *et al*., 2004).

Aflatoxins can have both acute and chronic effects to human health when ingested, inhaled, or absorbed through skin (Boonen *et al*., 2012; Reddy *et al*., 2010). Chronic exposure has been linked to HCC (aflatoxin is IARC group 1 carcinogen) (Gong *et al*., 2016; Liu *et al*., 2012; Zain, 2011), growth faltering in children (Gong *et al.*, 2003; Gong *et al*., 2004; Turner *et al*., 2007; Khlangwiset *et al*., 2011; Watson *et al*., 2015; Wild *et al*., 2015) by targeting the intestinal tract and inducing environmental enteropathy (Smith *et al*., 2012). Aflatoxins also impaired immunity in children (Gong *et al*., 2016; Jiang *et al*., 2005; Turner *et al*., 2003). Acute exposure known as aflatoxicosis can lead to vomiting, abdominal pain and liver failure (Zain, 2011), with documented fatality rates of 39% (Probst *et al*., 2007). Besides the health hazard, aflatoxins cause a trade barrier with significant economic impacts (Magan & Olsen, 2004).

Due to the adverse health impacts of aflatoxins, food used for consumption by humans and animals are routinely monitored and strictly regulated for aflatoxin contamination as part of standard food safety practices in most developed countries. However, in developing countries like Ethiopia, despite aflatoxin having a well characterized harmful effect on human health, understanding of human exposure and prevention has not been prioritised. Food shortages can intensify exposure to severely contaminated products.

Therefore, evaluating the levels and occurrences aflatoxin contamination in locally prepared cereal based CFs is paramount importance in protecting the health of the population particularly young children. Besides it is very important to provide evidence for safety interventions and regulations.

5.3 Objective

5.3.1 General objective

Determine the levels of total aflatoxin in CFs and their ingredients to see the occurrences and levels of contamination.

5.3.2 Specific objectives

- Determine the levels of total aflatoxins in the pre-milling cereals and legumes used for CFs preparation,
- Determine the levels of total aflatoxins in moldy cereals and legumes used for CFs preparation,
- Determine the levels of total aflatoxins in post production CFs and post production CFs stored for one month at households (HHs) and grain banks (GBs),
- Compare the occurrences of total aflatoxins in the post production CFs with post production CFs stored for one month at HHs and GBs.

5.4 Materials and Methods

5.4.1 Sampling areas

The samples were collected from two randmonly selected Villages in each 20 piloting Districts in Amhara, Tigray, Oromia, and SNNP regional states of Ethiopia. The details of the sampling areas were described in the materials and methods section of the previous chapter (Chapter 4).

5.4.2 Sample collection

Samples (moldy, pre-milling cereals and legumes, post production CFs, and post production CFs stored for one month at HHs and GBs) were collected from each two villages across all piloting Districts across all regions. The details of the sample collection protocol were described in the materials and methods section of the previous chapter (Chapter 4).

5.4.3 Experimental design

Completely randomized design (CRD) with two replications was used in all the experiments. Where the factors (types of cereals and legumes) are assigned completely at random, while analysing the levels of aflatoxins.

5.4.4 Moisture analysis

Moisture contents of the samples were determined according to AOAC (2000), using the official method 925.10. Dishes used for the moisture determination were first dried at 105^0C for one hour in drying oven. It then transferred to the desiccators cooled for 30 minutes, and weighed. The samples were mixed thoroughly and about 5 gm of the flour samples were transferred to the dried and weighed dishes. The dishes and their contents were placed in the drying oven and dried for 3 hrs at 105^0C, and then the dishes and their contents were cooled in desiccators to room temperature and reweighed.

Calculation:

$$MC = \left(\frac{w_2 - w_3}{w_2 - w_1}\right) \times 100$$

W_1 = mass of the dish, W_2 = mass of the dish and the sample before drying, and W_3 = mass of the dish and the sample after drying

5.4.5 Aflatoxin analysis

The levels of total aflatoxin were determined using competitive enzyme-linked immunoassay (ELISA) using the Agra Quant® ELISA aflatoxin, Art.No: COKAQ1000 commercial assay from Romer Labs (Romer Labs Singapore Pte. Ltd., Singapore) based on the method described by Iqbal *et al*. (2014) at CDC, USA. Total aflatoxins were extracted from 20 gm ground samples using 100 mL 70% methanol (70/30 (v/v) methanol/water) by blending for two min in a commercial blender (WARING commercial, California) and filtering through a Whatman one filter paper (Whatman TM International Ltd, Maid stone, England). Biopure, a naturally contaminated maize sample

(Romer Labs Diagnostic GmbH, Austria), was used as an external extraction control. The extracted samples (the filtrate), control standards (0, 4, 10, 20 and 40 µg/kg provided by kit) and conjugate (AFB1-horse radish peroxidase (HRP)) (provided by kit) were mixed and added to the antibody coated micro-wells and incubated for 15 min at room temperature. Then, the wells were washed 5 times with distilled water, the enzyme substrate (100 µL) (tetramethyl benzidine (TMB)) was added to the coated wells and allowed to incubate for 5 min to develop blue color, then a 100 µL stop solution (2N H_2SO_4) was added, changes the color from blue to yellow. Optical densities were measured using the SpectraMax250 microplate spectrophotometer with an absorbance filter of 450 nm and a differential filter of 630 nm. The intensity of the color (blue) was inversely proportional to the total aflatoxin concentration in the sample or standard. Results were calculated using Romer Log/Logit spread sheet provided by the manufacturer (that is, total aflatoxin contents in the tested samples were calculated using a standard curve obtained by the series of aflatoxin standards). Each sample was analyzed in duplicates. The average concentration was determined as a mean of two values and recorded in µg/kg.

Assay limit of detection (LOD): Is provided by the manufacturer and constitutes to 3 µg/kg for corn and other commodities and 5 µg/kg for sorghum.

Assessing assay reproducibility: Three independent extractions were performed from seven different samples and used to determine within and between assay variability's by calculating coefficient of variation (CV) using the following equation: Cv = Standard deviation/mean. Reproducibility results are shown in Table 5.1, 5.2 & 5.3 and demonstrate excellent within and between assay reproducibility.

Table 5.1 Within assay reproducibility

Sample No.	Run 1 (µg/kg)	Run 2 (µg/kg)	Mean(µg/kg)	SD	CV within assay
1	10.05	10.63	10.34	0.41	0.039
	13.00	13.67	13.33	0.48	0.036
	13.90	13.00	13.45	0.64	0.047
2	0.00	2.86	1.43	2.02	1.412
	1.22	1.27	1.24	0.04	0.032
	0.00	0.00	0.00	0.00	0.000
3	2.76	2.10	2.43	0.46	0.189
	1.30	2.77	2.04	1.04	0.510
	0.01	0.11	0.06	0.11	1.833
4	21.00	18.99	20.00	1.43	0.070
	17.17	17.74	17.46	0.40	0.022
	42.31	45.27	43.79	2.09	0.047
5	88.35	87.66	88.01	0.49	0.005
	99.13	99.82	99.47	0.49	0.004
	72.84	72.19	72.51	0.46	0.006
6	0.00	3.54	1.77	2.50	1.412
	0.55	0.00	0.27	0.38	1.407
	1.73	2.16	1.94	0.30	0.154
7	0.00	0.34	0.17	0.24	1.411
	1.96	3.67	2.82	1.21	0.429
	0.29	1.95	1.12	1.17	1.044

SD: Standard deviation, CV: Coefficient of variation.

Table 5.2 Between assay reproducibility

ID	Assay1	Assay2	Assay3	Mean (µg/kg)	SD	CV between assays
BP_STD	10.34	13.33	13.45	12.37	1.76	0.14
CDC_2	1.43	1.24	0.00	0.89	0.77	0.86
CDC_3	2.43	2.04	0.06	1.51	1.27	0.84
CDC_4	20.00	17.46	43.79	27.08	14.50	0.54
CDC_5	88.01	99.47	72.51	86.66	13.53	0.16
CDC_6	1.77	0.27	1.94	1.33	0.92	0.69
CDC_7	0.17	2.82	1.12	1.37	1.34	0.98

BP_STD: Biopure standard (contaminated maize), CDC: Centers for disease control and prevention, SD: Standard deviation, CV: Coefficient of variation.

Table 5.3 Summary reproducibility data

ID	CV within assay	CV between assays
BP_STD	0.040	0.14
CDC_2	0.481	0.86
CDC_3	0.844	0.84
CDC_4	0.046	0.54
CDC_5	0.005	0.16
CDC_6	0.991	0.69
CDC_7	0.961	0.98

BP_STD: Biopure standard (contaminated maize), CDC: Centers for disease control and prevention, CV: Coefficient of variation.

Assessing extraction efficiency: Extraction efficacy was evaluated using commercially available naturally contaminated maize samples (Romer Labs Diagnostic) and comparing experimental results with the known concentrations of aflatoxins in these samples. The extraction efficiency results are shown in Table 5.4 and demonstrate 94-122% recovery rates, which compares favorably with other studies.

Table 5.4 Percentage recovery

Sample	Original concentration (µg/kg)	Recovered concentration (µg/kg)	% recovery
Biopure naturally contaminated maize-1	11.04	10.34	94
Biopure naturally contaminated maize-2	11.04	13.33	120
Biopure naturally contaminated maize-3	11.04	13.45	122

5.4.6 Data analysis

The levels of total aflatoxins were analysed with analysis of variance (ANOVA) and paired sample t-test using SPSS 20 statistical software. When p values ($p < 0.05$) were found significant, Tukey was used for mean separation. The association between moisture content and aflatoxin levels were determined using pearson correlation.

5.5 Results and Discussion

5.5.1 Total aflatoxins in moldy cereals and legumes

Moldy cereals and legumes were used to prepare CFs during scarcity of ingredients in some parts of the piloting Districts. That is why it is necessary to evaluate the levels and occurrences of total aflatoxins in moldy cereals and legumes samples. Hence, a total of 20 moldy cereals and/or legumes samples were collected from all the regions and the levels of total aflatoxins were detected and the results are summarized in Table 5.5.

Table 5.5 Total aflatoxin contents of moldy cereals and legumes collected across all the regions

Region	District	Moldy Cereal/Legume	Moisture content (%)	Total aflatoxin (µg/kg)[a]
Amhara	Baso Liben	Teff	10.3	6.1 ± 0.76[c]
	Awabel	Maize	11.3	0.0 ± 0.00[a]
	Machakel	Wheat	10.3	6.0 ± 1.14[c]
	Burie	Wheat	9.30	3.4 ± 0.81[b]
	Sekela	Broad bean	8.10	2.9 ± 0.04[b]
Tigray	Endemekhoni	Wheat	11.9	6.2 ± 0.35[b]
	Atsbi Womberta	Maize	10.6	88.0 ± 0.49[c]
	Kilte Awlalo	Broad bean	8.74	5.8 ± 1.19[a]
	Samre Saharti	Maize	11.4	4.2 ± 0.32[a]
	Adwa	Teff	8.26	5.3 ± 0.20[a]
Oromia	Midega Tole	Sorghum	15.7	8.4 ± 1.69[b]
	Merti	Sorghum	11.8	4.4 ± 0.10[a]
	Sire	Haricot bean	9.72	6.0 ± 2.14[b]
	Kore	Maize	11.2	2.3 ± 3.20[a]
	Boset	Maize	10.9	20.0 ± 1.43[c]
SNNP	Geta	Barely	11.3	4.0 ± 0.41[a]
	Misrak Badwacho	Maize	12.7	8.0 ± 0.47[b]
	Aleta Chuko	Maize	11.2	4.0 ± 0.14[a]
	Shebedino	Maize	11.5	7.0 ± 0.86[b]
	Hula Tula	Maize	10.6	2.7 ± 0.55[a]

Means within each Region with different letters are significantly different ($p < 0.05$).

Total aflatoxins were detected in all of the moldy samples collected across all the regions except one sample from Amhara region, Awabel District. The concentration range of total aflatoxins found in Amhara, Tigray, Oromia and SNNP regions was 0.0 - 6.1 µg/kg, 4.2 - 88 µg/kg, 2.3 - 20 µg/kg and 2.7 - 8.0 µg/kg, respectively. The levels of total aflatoxins in moldy samples showed significant differences ($p < 0.05$) between Districts across all the regions. The differences in climatic conditions (Algul & Kara, 2014; Kaaya & Kyamuhangire, 2006), the moisture content in cereals and legumes (Alborch *et al*., 2012), storage and handling practices at the HHs and GBs might be the reason for the differences in the levels of aflatoxin contamination.

Although aflatoxins were detected in 19 out of 20 moldy samples collected across all the regions, only two of these samples exceeded the maximum limit (10 µg/kg) established by East African Community (EAC) (EAC, 2012) and nine samples exceeded the maximum limit (4 µg/kg) established by European Union (EU) for total aflatoxins in cereals and cereal products intended for direct human consumption (EU, 2003). This indicates that some of the molds might not be aflatoxin producers. Hence the samples are moldy but the fungi that spoil the samples may not produce aflatoxin. However, these results also indicate that the removal of moldy cereals and legumes during cleaning is an appropriate method for reducing aflatoxin contamination while preparing CFs (Kamala *et al*., 2016; Matumba *et al*., 2015).

5.5.2 Total aflatoxins in pre-milling cereals and legumes

The occurrence and levels of total aflatoxins in pre-milling cereals and legumes collected from Amhara region are presented in Table 5.6. Total aflatoxins were detected in all of the pre-milling cereals and legumes samples collected across all the five piloting Districts. The mean concentrations of total aflatoxins found were 3.0 µg/kg and 3.2 µg/kg in pre-milling cereals and legumes respectively. However, there is no significant difference ($p > 0.05$) in terms of total aflatoxins between pre-milling cereals and legumes.

In terms of total aflatoxin contamination among the five piloting Districts, Burie was the highest (with mean value of 4.3 µg/kg) while Sekela was the least (with mean value of 1.2 µg/kg) and Machakel was the highest (with mean value of 3.9 µg/kg) while Burie was the least (with mean values of 1.1 µg/kg) in pre-milling legumes samples, respectively. Although total aflatoxin was detected in all of the pre-milling cereals and legumes samples collected from Amhara region, but all the results were below the maximum limits (10 µg/kg) set by EAC for cereal flours intended for human consumption (EAC, 2012). However, seven out of 24 samples, surpassed the maximum limits (4 µg/kg) established by EU for total aflatoxins in cereals and cereal products intended for direct human consumption (EU, 2003).

Therefore, these results indicated that mothers/caregivers of young children and/or HEWs working at the GBs might be receiving moldy, insect infested and mechanicaly damaged cereals and legumes from mothers for CFs preparation. Moreover, they might not undertake good cleaning programs (sorting out mechanisms) while preparing pre-milling cereals and legumes. However, the moisture contents of all the pre-milling cereals and legumes were below 12%, which is acceptable in preventing mold spoilage and aflatoxin contamination. Since the moisture content is one of the key factors that affect mold spoilage and aflatoxin contamination in grains (Alborch *et al*., 2012). Thus, the practice of reducing the mositure content to acceptable levels before storage should be encouraged across the study areas.

Table 5.6 Total aflatoxin content of pre-milling cereals and legumes used for CFs production in Amhara region

Region	District	Cereal/Legume	Moisture content (%)	Total aflatoxin (µg/kg)[a]
Amhara	Baso Liben	Maize	9.66	1.0 ± 1.37[a]
		Wheat	9.32	3.3 ± 0.20[b]
		Teff	10.66	4.4 ± 0.28[b]
		Broad bean	10.1	3.1 ± 0.88[b]
		Pea	7.84	2.4 ± 0.24[a]
		Mean cereal	9.88	2.88 ± 1.69[x]
		Mean legume	8.97	2.79 ± 0.67[x]
	Awabel	Maize	9.68	2.2 ± 3.16[a]
		Wheat	9.58	2.5 ± 0.71[a]
		Teff	9.46	6.7 ± 0.37[b]
		Broad bean	9.82	2.5 ± 0.31[a]
		Chick pea	7.94	5.2 ± 0.95[b]
		Mean cereal	9.57	3.8 ± 2.66[x]
		Mean legume	8.88	3.87 ± 1.67[x]
	Machakel	Maize	9.16	0.6 ± 0.79[a]
		Wheat	8.48	3.9 ± 0.69[b]
		Teff	10.5	4.4 ± 0.28[b]
		Broad bean	7.36	4.6 ± 0.94[b]
		Pea	8.62	3.3 ± 0.53[b]
		Mean cereal	9.38	2.9± 1.91[x]
		Mean legume	8.00	3.9± 0.95[x]
	Burie	Maize	11.5	0.9 ± 1.33[a]
		Wheat	7.88	6.8 ± 0.12[b]
		Teff	9.80	5.1 ± 0.98[b]
		Broad bean	8.90	1.1 ± 1.17[a]
		Mean cereal	9.73	4.3 ± 2.78[y]
		Mean legume	8.90	1.1±1.17[x]
	Sekela	Maize	9.92	0.6 ± 0.36[a]
		Wheat	6.56	1.7 ± 0.15[a]
		Teff	12.3	1.2 ± 0.98[a]
		Broad bean	10.2	3.3 ± 0.75[b]
		Pea	9.86	3.3 ± 0.14[b]
		Mean cereal	9.59	1.2 ± 0.69[x]
		Mean legume	10.0	3.3 ± 0.44[y]
		All Districts mean of cereals	9.63	3.0 ± 2.22[m]
		All Districts mean of legumes	8.87	3.2 ± 1.28[m]

Means within each District with different letters are significantly different ($p < 0.05$)

Similarly, the levels and occurrences of total aflatoxins in the pre-milling cereals and legumes used for CFs production in Tigray region is depicted in Table 5.7. Total aflatoxins were detected in all of the pre-milling cereals and legumes samples collected across all the five piloting Districts. The mean concentrations of total aflatoxins found were 5.3 µg/kg and 3.1 µg/kg in pre-milling cereals and legumes, respectively. The levels of total aflatoxins showed significant differences ($p < 0.05$) between pre-milling cereals and legumes samples.

Table 5.7 Total aflatoxin content of pre-milling cereals and legumes used for CFs production in Tigray region

Region	District	Cereal/Legume	Moisture content (%)	Total aflatoxin (μg/kg)[a]
Tigray	Endemekhoni	Wheat	7.18	1.8 ± 0.23^{a}
		Maize	8.98	6.7 ± 3.71^{a}
		Pea	8.66	3.3 ± 0.62^{a}
		Mean of cereals	8.08	4.2 ± 3.54^{x}
		Mean of legumes	8.66	3.3 ± 0.62^{x}
	Atsbi Womberta	Wheat	10.9	1.5 ± 0.46^{a}
		Maize	10.8	4.8 ± 0.20^{b}
		Pea	9.58	2.5 ± 0.57^{a}
		Mean of cereals	10.9	3.1 ± 1.94^{x}
		Mean of legumes	9.58	2.5 ± 0.57^{x}
	Kilte Awlalo	Wheat	6.44	9.9 ± 0.24^{c}
		Teff	11.5	3.8 ± 0.21^{b}
		Broad bean	8.98	2.9 ± 0.08^{a}
		Mean of cereals	8.97	6.9 ± 3.57^{x}
		Mean of legumes	8.98	2.9 ± 0.08^{x}
	Samre Saharti	Wheat	8.48	7.5 ± 1.37^{b}
		Maize	9.14	3.6 ± 0.35^{a}
		Broad bean	8.60	4.5 ± 0.46^{a}
		Mean of cereals	8.81	5.6 ± 2.38^{x}
		Mean of legumes	8.60	4.5 ± 0.46^{x}
	Adwa	Wheat	7.60	9.2 ± 0.40^{c}
		Teff	9.46	4.1 ± 0.46^{b}
		Broad bean	8.58	2.3 ± 0.69^{a}
		Mean of cereals	8.53	6.6 ± 2.97^{y}
		Mean of legumes	8.58	2.3 ± 0.69^{x}
	All Districts mean of cereals		9.06	5.3 ± 3.00^{y}
	All Districts mean of legumes		8.88	3.1 ± 0.92^{x}

Means within each District with different letters are significantly different ($p < 0.05$)

From Table 5.7 it can be shown that, pre-milling cereals had the highest range of total aflatoxin occurrence (3.1 - 6.9 μg/kg) than pre-milling legumes (2.3 - 4.5 μg/kg). All the pre-milling samples collected from Tigray region presented detectable levels of total aflatoxins, although neither the mean nor the highest values obtained in the analyzed samples exceeded the maximum limit (10 μg/kg) (EAC, 2012). However, six out of 15 samples exceeded the maximum limit (4 μg/kg) established by EU for total aflatoxins in cereals and cereal products intended for direct human consumption (EU, 2003). Although some of these results exceeded the EU limits, it is consistent with a previous study by Algul & Kara (2014) reporting a range of total aflatoxin (0.13 – 13.92 µg/kg) in maize flour collected from Turkey. Another study by Kimanya *et al*. (2008) also reported up to 158 µg/kg of total aflatoxin in maize samples used for direct human consumption in rural villages of Tanzania.

Pre-milling samples collected from Oromia region were analysed for total aflatoxins and the results are depicted in Table 5.8. Total aflatoxins were detected in 13 out of 15 pre-milling samples collected across all the five Districts. The mean concentrations of total aflatoxins found were 2.2 µg/kg and 4.2 µg/kg in pre-milling cereals and legumes samples, respectively. The levels of total aflatoxins showed significant differences ($p < 0.05$) between pre-milling cereals and legumes samples. None of the results exceeded the maximum limit (10 µg/kg) (EAC, 2012). However, three out of 15 samples exceeded the maximum limit (4 µg/kg) established by the EU for total aflatoxins in cereals and cereal products intended for direct human consumption (EU, 2003). Although, these results were lower than a previous study by Kos *et al*. (2014) reporting a mean range of total aflatoxins (1.05 – 70.03 µg/kg) in maize samples collected from Serbia.

Table 5.8 Total aflatoxin content of pre-milling cereals and legumes used for CFs production in Oromia region

Region	District	Cereal/Legume	Moisture content (%)	Total aflatoxin (μg/kg)[a]
Oromia	Midega Tole	Sorghum	9.18	1.2 ± 0.61[a]
		Maize	9.02	3.2 ± 0.49[a]
		pea	6.64	2.1 ± 0.83[a]
		Mean of cereal	9.10	2.2 ± 1.19[x]
		Mean of legume	6.64	2.1 ± 0.83[x]
	Merti	Sorghum	9.08	0.0 ± 0.00[a]
		Wheat	8.06	0.6 ± 0.47[a]
		Broad bean	6.86	5.8 ± 0.65[b]
		Mean of cereal	8.57	0.3 ± 0.43[x]
		Mean of legume	6.86	5.8 ± 0.65[y]
	Sire	Wheat	8.32	3.4 ± 0.25[a]
		Maize	8.80	3.4 ± 1.24[a]
		Haricot bean	7.82	2.7 ± 0.57[a]
		Mean of cereal	8.56	3.4 ± 0.73[x]
		Mean of legume	7.82	2.7 ± 0.57[x]
	Kore	Barley	9.52	2.5 ± 0.05[b]
		Maize	10.8	0.0 ± 0.00[a]
		Broad bean	6.78	7.8 ± 0.28[c]
		Mean of cereal	10.2	1.3 ± 1.46[x]
		Mean of legume	6.78	7.8 ± 0.28[y]
	Boset	Teff	8.62	5.9 ± 0.43[b]
		Maize	9.22	2.2 ± 0.15[a]
		Chick pea	6.76	2.7 ± 1.26[a]
		Mean of cereal	8.92	4.0 ± 2.18[x]
		Mean of legume	6.76	2.7 ± 1.26[x]
	All District mean of cereals		9.07	2.2 ± 1.84[m]
	All District mean of legumes		6.97	4.2 ± 2.40[n]

Means within each District with different letters are significantly different ($p < 0.05$)

Similarly, the levels of total aflatoxin contamination in pre-milling cereals and legumes used for CFs production in SNNP region is depicted in Table 5.9. Total aflatoxins were detected in 10 out of 12 pre-milling samples collected across all the five Districts. The mean concentrations of total aflatoxins in pre-milling cereals and legumes found respectively were 1.7 μg/kg and 3.9 μg/kg. The levels of total aflatoxins showed significant differences ($p < 0.05$) between pre-milling cereals and legumes samples. Total aflatoxin was detected in the majority of the pre-milling samples collected from SNNP region, but none of the results exceeded the maximum limit (10 μg/kg) (EAC, 2012). However, two samples exceeded the maximum limit (4 μg/kg) established by EU for total aflatoxins in cereals and cereal products intended for direct human consumption (EU, 2003).

Table 5.9 Total aflatoxin content of pre-milling cereals and legumes used for CFs production in SNNP region

Region	District	Cereal/Legume	Moisture content (%)	Total aflatoxin (µg/kg)[a]
SNNP	Geta	Barely	6.80	0.0 ± 0.0[a]
		Wheat	7.34	0.9 ± 1.23[a]
		Broad bean	8.72	1.8 ± 0.46[ab]
		Pea	7.80	3.9 ± 1.57[b]
		Mean of cereal	7.07	0.9 ± 1.1[x]
		Mean of legume	8.26	3.2 ± 1.27[y]
	Misrak Badwacho	Maize	7.84	0.0 ± 0.00[a]
		Haricot bean	6.70	4.1 ± 1.79[a]
		Mean of cereal	7.84	0.0 ± 0.00[x]
		Mean of legume	6.70	4.1 ± 1.79[x]
	Aleta Chucko	Maize	9.50	4.5 ± 0.23[a]
		Haricot bean	6.74	3.1 ± 2.59[a]
		Mean of cereal	9.50	4.5 ± 0.23[x]
		Mean of legume	6.74	3.1 ± 2.59[x]
	Shebedino	Maize	9.50	1.9 ± 0.30[a]
		Haricot bean	6.90	3.2 ± 1.14[a]
		Mean of cereal	9.50	1.9 ± 0.30[x]
		Mean of legume	6.90	3.2 ± 1.14[x]
	Hula Tula	Maize	8.40	2.8 ± 0.23[a]
		Haricot bean	6.82	7.3 ± 0.17[b]
		Mean of cereal	8.40	2.8 ± 0.23[x]
		Mean oflegume	6.82	7.3 ± 0.17[y]
		All Districts mean of cereals	8.46	1.7 ± 1.74[m]
		All Districts mean of legumes	7.08	3.9 ± 2.08[n]

Means within each District with different letters are significantly different ($p < 0.05$)

Over all, the levels of total aflatoxin in all the pre-milling cereals and legumes samples collected across all the regions were below the maximum limit (10 µg/kg) established by EAC for cereal flours intended for human consumption. However, 19 out of 66 samples (29%) exceeded the maximum limit (4 µg/kg) established by EU for total aflatoxins in cereals and cereal products intended for direct human consumption. Further, all the legumes samples collected across all the regions are contaminated by aflatoxin like that of cereals.

5.5.3 Total aflatoxins in CFs

The occurrence and levels of total aflatoxins in the post-production CFs, post-production one-month CFs stored at HHs (CFs (HH)) and GBs (CFs (GB)) collected from Amhara, Tigray, Oromia, and SNNP regions are summarized in Table 5.10. Total aflatoxins were detected in 59 out of 60 CFs collected across all the regions. The mean concentrations of total aflatoxins of the entire regions found were 3.8 µg/kg, 7.0 µg/kg and 7.1 µg/kg in post-production CFs, CFs (HH) and CFs (GB), respectively. The levels of total aflatoxins showed significant differences ($p < 0.05$) between post-production CFs, CFs (HH) and CFs (GB) in the entire regions.

Total aflatoxins were detected in all of the CFs collected across all the five Districts in Amhara region. The mean levels of total aflatoxins found were 3.1 µg/kg, 8.1 µg/kg and 7.7 µg/kg in post-production CFs, CFs (HH) and CFs (GB), respectively. The levels of total aflatoxins showed significant differences ($p < 0.05$) between post-production CFs, CFs (HH) and CFs (GB).

Although aflatoxin was detected in all CFs, only one sample exceeded the maximum limit (10 µg/kg) established by Kenya Bureau of Standards (KEBS) (KEBS, 2013) for total aflatoxins in cereal-based CFs for older infants and young children. Further, our results are consistent with a previous study by Rushunju *et al*. (2013) who reported the levels of total aflatoxin (range, 8.4 - 11 µg/kg) in locally produced cereal-based CFs in Tanzania. Another study by Razzazi-Fazeli *et al*. (2004) also reported detectable levels of total aflatoxin (range, 5.8 – 12.4 µg/kg) in maize-based CFs in Indonesia. A similar study by Matumba *et al*. (2014) reported total aflatoxin levels (range, 0.5 -10.4 µg/kg) in maize-based baby foods in Malawi.

Table 5.10 Total aflatoxin content of post production CFs and CFs stored at households (HHs) and grain banks (GBs) for one month

Region	District	Cereal/Legume combinations	Moisture content (%) and total aflatoxin (μg/kg)					
			Post-production CFs		CFs(HH)		CFs(GB)	
			Moisture (%)	Total aflatoxin (μg/kg)^{a}	Moisture (%)	Total aflatoxin (μg/kg)^{a}	Moisture (%)	Total aflatoxin (μg/kg)^{a}
Amhara	Baso Liben	M/W/T/BB/P	9.02	7.3 ± 0.38^{b}	8.16	10.0 ± 0.09^{bc}	8.02	5.9 ± 0.44^{a}
	Awabel	M/W/T/BB/P	8.54	0.5 ± 0.75^{a}	8.29	8.3 ± 0.64^{b}	8.19	8.3 ± 1.30^{ab}
	Machakel	M/W/T/BB/CP	9.48	4.6 ± 1.16^{a}	9.53	11.3 ± 1.14^{c}	9.43	10.0 ± 0.56^{b}
	Burie	M/W/T/BB	8.84	2.1 ± 1.80^{a}	8.23	7.4 ± 1.31^{b}	7.89	5.5 ± 1.56^{a}
	Sekela	M/W/T/BB/P	10.2	1.1 ± 0.89^{a}	10.7	3.6 ± 0.43^{a}	9.97	8.8 ± 0.47^{ab}
	Mean		**9.22**	**3.1 ± 2.81^{x}**	**8.98**	**8.1 ± 2.94^{y}**	**8.70**	**7.7 ± 1.93^{y}**
Tigray	Endemekhoni	W/M/P	7.94	1.7 ± 0.05^{a}	8.03	4.6 ± 0.42^{a}	7.68	1.2 ± 1.76^{a}
	Atsbi Womberta	W/M/P	9.22	0.0 ± 0.00^{a}	9.37	4.6 ± 0.17^{a}	9.25	4.9 ± 0.43^{b}
	Kilte Awlalo	W/T/BB	7.98	4.1 ± 0.82^{b}	7.86	4.4 ± 0.43^{a}	8.06	4.4 ± 0.77^{b}
	Samre Saharti	W/M/BB	8.12	8.0 ± 0.03^{c}	8.34	8.4 ± 3.03^{ab}	8.46	4.1 ± 0.41^{ab}
	Adwa	W/T/BB	9.50	3.5 ± 0.10^{b}	9.60	9.9 ± 1.51^{b}	10.0	8.4 ± 1.34^{c}
	Mean		**8.55**	**3.5 ± 3.00^{x}**	**8.64**	**6.4 ± 2.58^{x}**	**8.69**	**4.6 ± 2.57^{x}**
Oromia	Midega Tole	S/M/P	7.22	4.0 ± 0.32^{a}	6.73	7.7 ± 0.88^{a}	6.92	10.0 ± 0.18^{c}
	Merti	S/W/BB	7.00	3.5 ± 0.33^{a}	7.93	10.4 ± 1.09^{b}	8.01	12.4 ± 0.65^{c}
	Sire	W/M/HB	7.64	8.0 ± 0.63^{b}	7.41	7.0 ± 0.98^{a}	7.25	0.2 ± 0.28^{a}
	Kore	B/M/BB	8.36	2.2 ± 1.69^{a}	8.89	5.5 ± 0.39^{a}	8.74	9.0 ± 1.87^{c}
	Boset	M/T/CP	7.28	1.2 ± 1.64^{a}	6.98	6.3 ± 0.55^{a}	7.24	6.0 ± 0.85^{b}
	Mean		**7.50**	**3.8 ± 2.60^{x}**	**7.59**	**7.4 ± 1.88^{x}**	**7.63**	**7.5 ± 4.69^{x}**
SNNP	Geta	B/W/BB/P	5.92	4.8 ± 1.65^{b}	6.59	6.7 ± 0.37^{a}	6.63	9.7 ± 1.89^{a}
	Misrak Badwacho	M/HB	7.82	6.9 ± 1.03^{bc}	10.5	5.4 ± 0.47^{a}	8.16	9.8 ± 0.11^{a}
	Aleta Chuko	M/HB	7.46	5.3 ± 0.16^{ab}	8.25	3.8 ± 1.12^{a}	7.36	6.8 ± 1.75^{a}
	Shebedino	M/HB	7.82	2.9 ± 0.10^{ab}	8.18	7.4 ± 2.58 ^{a}	8.14	9.4 ± 1.61^{a}
	Hula Tula	M/HB	7.16	1.5 ± 0.57^{a}	8.13	7.0 ± 1.35^{a}	7.73	7.6 ± 0.38^{a}
	Mean		**7.24**	**4.3 ± 2.11^{x}**	**8.33**	**6.1 ± 1.47^{x}**	**7.60**	**8.7 ± 1.37^{y}**
	All Regions Mean		**8.13**	**3.7 ± 0.49^{m}**	**8.39**	**7.0 ± 0.94^{n}**	**8.16**	**7.1 ± 1.75^{n}**

Means in the same columns and in the same rows not followed by the same letters are significantly different ($p < 0.05$). Abbreviations: M - Maize, W - Wheat, T - Teff, BB - Broad bean, P - Pea, CP - Chick pea, S - Sorghum, HB - Haricot bean, and B - Barley.

Generally, the levels of aflatoxin contamination increased in CFs from post-production to following one-month storage HHs and GBs across all the Districts in the region. This might be due to the effects of the storage time (Kaaya & Kyamuhangire, 2006), storage structures and the storage environments. Further, our results are consistent with a previous study by Saleemullah *et al*. (2006) reported the increment of total aflatoxins levels both in cereals and cereal-based products during two to three months' storage periods. Another study by Iqbal *et al*. (2006) also reported the increment of total aflatoxins levels both in cereals and cereal based products during two to three months' storage periods.

Similarly, total aflatoxins were detected in CFs collected across all the five Districts in Tigray region (Table 5.10). The mean levels of total aflatoxins found were 3.5 µg/kg, 6.4 µg/kg and 4.6 µg/kg in post-production CFs, CFs (HH) and CFs (GB), respectively. However, the levels of total aflatoxins showed non-significant differences ($p > 0.05$) between post-production CFs, CFs (HH) and CFs (GB) in the entire region, but there are significant differences ($p < 0.05$) among Districts.

Aflatoxins were detected in 14 out of 15 CFs collected from Tigray region. However, none of the results exceeded the maximum limit (10 µg/kg) established by KEBS (KEBS, 2013) for total aflatoxins in cereal-based CFs for older infants and young children. Further, our results are consistent with a previous study by Blankson & Mill-Robertson (2016) who reported total aflatoxin levels (range, 1.41 to 8.78 µg/kg) in cereal-legume based CFs in Ghana. Another study by Amin *et al*. (2010) also reported less than 10 µg/kg aflatoxin levels in cereal-based baby foods collected in Egypt.

Total aflatoxins were also detected in all of the CFs collected across all the five Districts in Oromia region and the results are also indicated in (Table 5.10). The mean levels of total aflatoxins found in the entire region were 3.8 µg/kg, 7.4 µg/kg and 7.5 µg/kg in post-production CFs, CFs (HH) and CFs (GB), respectively. However, the levels of total aflatoxins showed non-significant differences ($p > 0.05$) between post-production CFs, CFs (HH) and CFs (GB). But among piloting Districts, there is significant differences ($p < 0.05$) in terms of CFs, CFs (HH) and CFs (GB).

Total aflatoxins were detected in all CFs collected from Oromia region. However, only two of the samples exceeded the maximum limit (10 µg/kg) established by KEBS (KEBS, 2013) for total aflatoxins in cereal-based CFs for older infants and young children. Although two of our results surpassed the maximum limits established by KEBS, it is consistent with a previous study by Kimanya *et al*. (2014) reporting detectable levels of total aflatoxin (range, 0.11 - 386 µg/kg) in maize-based CFs in Tanzania. Another study by Geary *et al*. (2016) reported total aflatoxin levels (mean range, 0.2- 38.22 µg/kg) in maize-based porridges in Tanzania. Razzazi-Fazeli *et al*. (2004) also reported total aflatoxin levels (range, 5.8 - 12.4 µg/kg) in maize-based CFs in Indonesia. A similar study in North Africa by Aidoo *et al*. (2011) reported total aflatoxin levels (mean value of 19 µg/kg) in cereal-based infant foods.

Similarly, total aflatoxins were detected in all of the CFs collected from the SNNP region (Table 5.10). The mean concentrations of total aflatoxins found were 4.3 µg/kg, 6.1 µg/kg and 8.7 µg/kg in post-production CFs, CFs (HH) and CFs (GB), respectively. The levels of total aflatoxins showed significant differences ($p < 0.05$) between post-production CFs, CFs (HH) and CFs (GB).

Although aflatoxins were detected in all of the CFs samples collected from SNNP region, but, none of the results exceeded the maximum limit (10 µg/kg) established by KEBS (KEBS, 2013) for total aflatoxins in cereal based CFs for older infants and young children. Besides, our results are consistent with a previous study in Egypt by Amin *et al*. (2010) who reported less than 10 µg/kg total aflatoxin levels in cereal based baby foods. Another study by Blankson & Mill-Robertson (2016) also reported total aflatoxin levels (mean range, 1.41 to 8.78 µg/kg) in cereal-legume based CFs in Ghana.

Overall, there is a significant increment ($p < 0.05$) in the mean levels of total aflatoxin in CFs from post-production to following one-month storage in HHs and GBs in all regions (Figure 5.1).

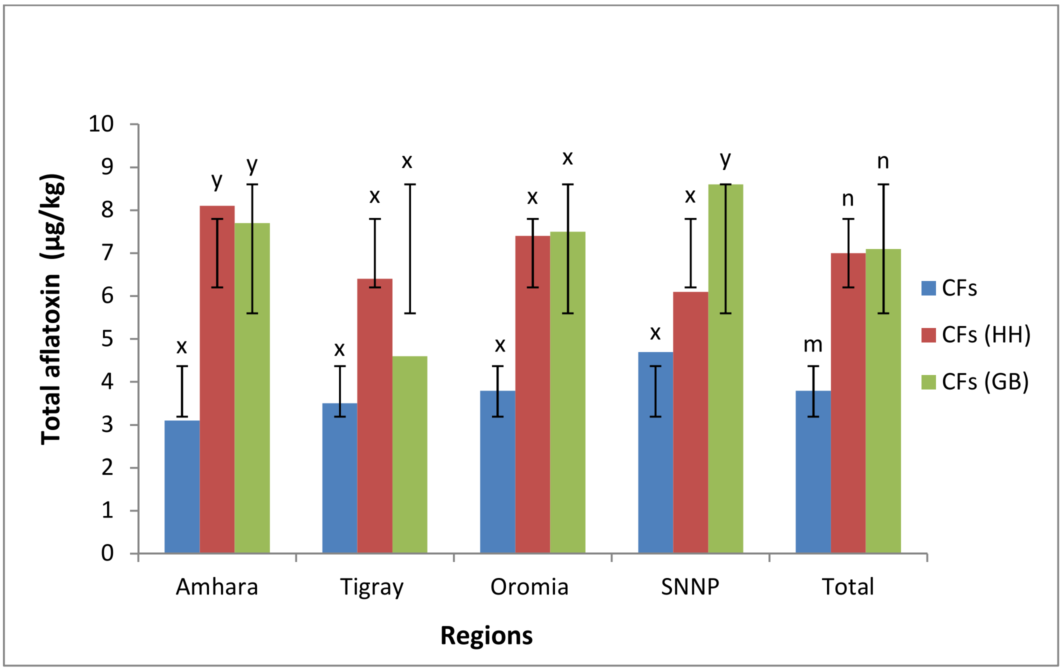

Figure 5.1 The variation in the mean levels of total aflatoxins in post production CFs, CFs (HH) and CFs (GB) across all regions

5.5.4 Correlation between moisture content and levels of aflatoxin in moldy, pre-milling and CFs

Except one sample (sorghum moldy sample collected from Midega Tole District, moisture content, 15.7%) the moisture content of all the samples was less than or equal to 12%. This is acceptable in preventing the risks of mold and aflatoxin contamination (Alborch *et al*., 2012). Over all, the moisture content of the pre-milling ingredients and the levels of total aflatoxins were found to have a negative correlation by Pearson correlation (r: -0.253 and $p = 0.042$). However, the moisture content of moldy, post-production CFs, CFs (HH) and CFs (GB) samples collected across all the regions showed no statistically significant correlation with the levels of total aflatoxins ($p > 0.05$). Previous studies (Lahouar *et al*., 2016; Mousa *et al*., 2016) showed a positive correlation between moisture content (particularly water activity) and aflatoxin contamination in cereals. However, our results are not consistent with those studies, probably because the moisture content of our samples was below the acceptable level (12%).

5.6 Conclusion

The results of this study confirm that aflatoxins are widespread contaminants of cereals and legumes intended for human consumption. Aflatoxins were detected in 140 out of 146 samples, but, only five samples exceeded a toxic level (10 µg/kg). The levels and occurrences of total flatoxin were higher in moldy samples but lower in pre-milling cereals and legumes. The total aflatoxin levels increased in CFs from post-production to following one-month storage in HHs and GBs in all regions. This clearly indicates that storage time is a risk factor for mold growth and aflatoxin contamination in CFs. Although, aflatoxin levels were considered safe for consumption in most samples, more efforts should be implemented to reduce the contamination level, particularly as these CFs are intended for consumption by young children. Education and other support must continue to identify, remove and prevent proliferation of aflatoxin and other mycotoxins in the CFs produced in these programs.

CHAPTER 6. ASSESSMENT OF AFLATOXIN EXPOSURE AMONG YOUNG CHILDREN IN ETHIOPIA USING URINARY BIOMARKERS

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ARTICLE

Assessment of aflatoxin exposure among young children in Ethiopia using urinary biomarkers

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ABSTRACT

The direct measurement of biomarkers of exposure in biological fluids such as urine has become important for assessing aflatoxin exposure in humans as it is the only tool that integrates exposures from various routes. For this reason, a study was conducted to assess aflatoxin exposure among young children in Ethiopia using urinary biomarkers. A cross-sectional study was conducted in ten Woredas (Districts) from Amhara and Tigray regional states of Ethiopia including 200 children (aged 1–4 years). A total of 200 urine samples were collected from 200 children and assessed for the levels of aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2) and aflatoxin M_1 (AFM_1) using a validated LC-MS/MS method. Aflatoxins were detected in 34/200 (17%) of the urine samples whereby four out of five analysed aflatoxins were detected. AFM_1 was detected in 14/200 (7%) of the urine samples in a range of 0.06–0.07 ng/mL. AFB_2, AFG_2 and AFG_1 were detected in respectively 9/200 (4.5%), 6/200 (3%) and 5/200 (2.5%) of the urine samples whereas AFB_1 was not detected in any of the samples. In this study, there was no association between the different malnutrition categories (stunted, wasting and underweight) and aflatoxin exposure. However, the biomarker analysis showed a clear exposure of young children to aflatoxins. Therefore, awareness to the public is important to prevent potential health consequences of aflatoxins.

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Biomarker; urine; aflatoxin; children; exposure assessment

Introduction

Exposure to environmental factors during early childhood is considered to be critical for growth and the development of diseases in later life (Terry and Susser 2001; Polychronaki et al. 2008). Young children are vulnerable to environmental toxicants because of their greater relative exposure (greater dietary intake per kg body weight in comparison with adults), immature metabolism and elimination, and higher rates of growth and development (Makri et al. 2004). In addition to physiologic vulnerabilities, young children may have great social vulnerabilities as well as poverty and malnutrition. In developing countries, especially in West Africa and Sub-Saharan Africa (SSA), many children are not only malnourished, but they are also frequently exposed to high levels of toxic fungal metabolites (aflatoxins) in their diet (Cardwell and Henry 2004; Turner et al. 2000, 2003, 2012; Wang et al. 2001).

For the past decades, aflatoxin exposure in humans has been assessed by quantifying the presence of these contaminants in food commodities. This approach lacks accuracy since aflatoxins are heterogeneously distributed within a given food (Kuiper-Goodman 1999). Second, humans can be exposed to aflatoxins through other routes like physical contact via the skin and inhalation (Boonen et al. 2012). Third, there are individual variations in terms of absorption, distribution, metabolism and excretion (Arcella and Leclercq 2004). Fourth, sometimes the food is no longer available for analysis in case of disease outbreak for which aflatoxin exposure was suspected. As a result, it cannot reflect accurate information on the individual exposure of aflatoxins.

In order to sidestep the above-mentioned limitations, biomarkers have been proposed as a suitable alternative. Biomarkers in biological fluids such as blood and urine are quantitative dosimeters of individual exposure, as well as initial cautioning signs of health effects (Turner et al. 2000; Wild and Gong

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6.1 Abstract

The direct measurement of biomarkers of exposure in biological fluids such as urine has become an added value to assess aflatoxin exposure in humans as it is the only tool that integrates the exposure from various routes. For this reason, a study was conducted to assess aflatoxin exposure among young children from Ethiopia using urinary biomarkers. A cross-sectional study was conducted in ten Districts from Amhara and Tigray regional states of Ethiopia including 200 children (aged 1- 4 years). A total of 200 urine samples were collected from 200 children and assessed for the levels of aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2) and aflatoxin M1 (AFM1) using a validated LC-MS/MS method. Aflatoxins were detected in 34/200 (17%) of the urine samples whereby four out of five analyzed aflatoxins were detected. AFM1 was detected in 14/200 (7%) of the urine samples in a range of 0.06-0.07 ng/mL. AFB2, AFG2 and AFG1 were detected in respectively 9/200 (4.5%), 6/200 (3%) and 5/200 (2.5%) of the urine samples whereas AFB1 was not detected in any of the samples. In this study, there was no association between the different malnutrition categories (stunted, wasting and underweight) and aflatoxin exposure. However, the biomarker analysis showed a clear exposure of young children to aflatoxins. Therefore, awareness to the public is important to prevent potential health consequences of aflatoxins.

Keywords: Biomarker; Urine; Aflatoxin; Children; Exposure assessment

6.2 Introduction

Early childhood environment is considered to be critical for growth and risk of disease in later life (Polychronaki *et al*., 2008; Terry & Susser, 2001). Young children are vulnerable to environmental toxicants because of their greater relative exposure (greater dietary intake than adults), immature metabolism, and elimination, and higher rates of growth and development (Makri *et al*., 2004). In addition to physiologic vulnerabilities, young children may have great social vulnerabilities as well as poverty and malnutrition. In developing countries, especially in West Africa and Sub-Saharan Africa (SSA), many children are not only malnourished, but they are also frequently exposed to high levels of toxic fungal metabolites (aflatoxins) in their diet (Cardwell & Henry, 2004; Turner *et al*., 2000; Turner *et al*., 2003; Turner *et al*., 2012; Wang *et al*., 2001).

For the past decades, aflatoxin exposure in humans has been assessed by detecting and quantifying the presence of this contaminant in food commodities. This indirect approach is generally considered less accurate since aflatoxins are heterogeneously distributed with in a given food and marked seasonal variations (Kuiper-Goodman, 1999). Secondly, humans can be exposed to aflatoxins through other routes like physical contact via the skin and inhalation. Thirdly, there are variations among individuals in terms of absorption, distribution, metabolism and excretion of the toxins (Arcella & Leclercq, 2004). Fourthly, sometimes the food is no longer available for analysis in case of disease outbreak for which aflatoxin exposure was suspected. As a result, it cannot reflect accurate information on the individual exposure of aflatoxins.

In order to sidestep the above-mentioned limitations, biomarkers have been proposed as a suitable alternative. Biomarkers in biological fluids such as blood and urine have the possible to be quantitative dosimeters of individual exposure, as well as initial cautioning signs of health effects (Turner *et al*., 2000; Wild & Gong, 2010). Urinary aflatoxin biomarkers of exposure and effect for aflatoxins have been validated in comprehensive studies in animals (Song *et al.*, 2013) and humans (Abia *et al*., 2013; Ediage *et al.*, 2012; Ediage *et al.*, 2013; Ezekiel *et al.*, 2014; Mykkane *et al.*, 2005; Piekkola *et al.*, 2012). The use of aflatoxin as biomarkers reflects not only the dietary exposure of the individual but also the uptake, toxicokinetics and toxicodynamics (Ediage *et al.*, 2012; Polychronaki *et al*., 2008).

Aflatoxin B1 (AFB1) and its metabolites are excreted through urinary routes (Abia *et al*., 2013; Ali *et al*., 2016; Ediage *et al*., 2013; Ezekiel *et al*., 2014; Mykkanen *et al*., 2005; Piekkola *et al*., 2012). The aflatoxin metabolite, aflatoxin M1 (AFM1), is also excreted in breast milk if the mother is exposed to aflatoxin contaminated foods (Adejumo *et al*., 2013; Atasever *et al*., 2014; Iha *et al*., 2014; Polychronaki *et al*., 2006; Polychronaki *et al*., 2007). Besides, aflatoxin-albumin adduct is usually the target metabolite in blood for aflatoxin exposure (Anitha *et al*., 2014; Jolly *et al*., 2015; Schleicher *et al*., 2013; Sharma & Farmer, 2004; Tang *et al*., 2008; Watson *et al*., 2016). However, obtaining blood samples from younger children creates a high barrier for participation. As a result, urine is chosen as a target matrix for this study.

Aflatoxin exposure has been associated with growth faltering (Gong *et al*., 2002; Gong *et al*., 2004; IARC, 2016; Polychronaki *et al*., 2008; Turner *et al*., 2007; Turner, 2013; Wild *et al*., 2015) and immune suppression in young children (Turner *et al*., 2003). Early life exposures could also be a contributing factor towards the early onset of HCC (Gong *et al*., 2016; Kirk *et al*., 2005; Omer *et al*., 2004; Polychronaki *et al*., 2008; Wang *et al*., 2001; Wild & Gong, 2010).

As a result, we did knowledge and practice of the mothers about the safe processing and storage of CFs (Beyene *et al*., 2016). In addition, we have been evaluating the levels of aflatoxins from the CFs and their ingredients. Therefore, as a continuation of these works, the aim of this epidemiological study was to assess aflatoxin exposure among young children through the determination of urinary aflatoxin; AFB1, aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), and AFM1 using a validated LC-MS/MS method. Additionally, we investigated the relationship between the different malnutrition categories and aflatoxin exposure.

6.3 Objective

6.3.1 General objective

Assess aflatoxin exposure among young children using urinary biomarkers.

6.3.2 Specific onjectives

- Identify the possible routes of aflatoxin exposure of the young children using Food Frequency Questionnaire (FFQ),
- Determine the anthropometric measurements of the young children to see the possible correlation with the levels of urinary aflatoxin,
- Determine the levels of urinary aflatoxin (AFB1, AFB2, AFG1, AFG2, and AFM1).

6.4 Materials and methods

6.4.1 Study areas

The study was conducted in ten Districts in Amhara and Tigray regional states of Ethiopia. Amhara and Tigray regions are the most performing regions interms of bartering of CFs. Further, the levels of aflatoxin in the CFs and their ingredients collected from these two regions are relatively high. As a result, Amhara and Tigray regions are purposively selected out of the four regions for the biomarker study. In each District, two villages were chosen according to the study protocol developed in a previous study (Chapter 4) (where the CFs and their ingredients were collected for aflatoxin and microbial analysis).

6.4.2 Participant recruitment

Ethical approval and clearance for this study was obtained from the National Research Ethics Review Committee (NRERC) of the Ethiopian Ministry of Science and Technology and from AAU, College of Natural Sciences Research Ethics Review Committee (Annex C). And approval was also obtained from Amhara and Tigray regional health bureau review board. After which, written permission was communicated for the respective District health offices and health extension workers (HEW) in each village. Twenty children per District (in total 200 children, aged 1-4 years), were recruited with the inclusion of one child per household. The purpose of the study was thoroughly explained to each individual respondent (mothers/caregivers) and written consent was obtained before administrating FFQ, anthropometric measurements and collecting urine samples. The fieldwork took place in January 2016.

6.4.3 Questionnaire

A food frequency questionnaire (FFQ) was administered by trained interviewers (nurses) (Annex D). The food consumption details of the young children and frequency of consumption on weekly bases were recorded in order to identify the possible route of aflatoxin contamination or exposure. Additionally, information such as age, gender and weaning status of the children was collected. The age of each child was collected based on the record found at the health post with the support of HEWs.

6.4.4 Anthropometric measurements

The weight, height, and mid upper arm circumference (MUAC) of all the young children were measured using accurately calibrated instruments. From the collected anthropometric data three indices of nutritional status - namely the weight-for- age Z-score (WAZ), the height-for-age Z-score (HAZ) and weight-for-height Z-score (WHZ) were performed based on the growth standards published by the World Health Organization (WHO) in 2006.

6.4.5 Urine sample collection

Morning urine samples were demanded from all volunteers. The mothers/caregivers were informed a day prior to the day of sample collection to come to the health post with their children at 8 AM. The urine samples were collected on spot at the health post using an urine collection bag (Figure 6.1). Approximately 40 ml urine was collected from each child and immediately transferred to a urine cup and stored at -18 ^{0}C using a portable freezer (Figure 6.2). All urine samples were transported to AAU and stored at -18 ^{0}C until shipment. Due to lack of laboratory facilities in Ethiopia to determine urinary aflatoxin, all samples (15 ml each) were preserved on dry ice and transported to the Laboratory of Food Analysis, Ghent University, Belgium.

Figure 6.1 Urine collection bag

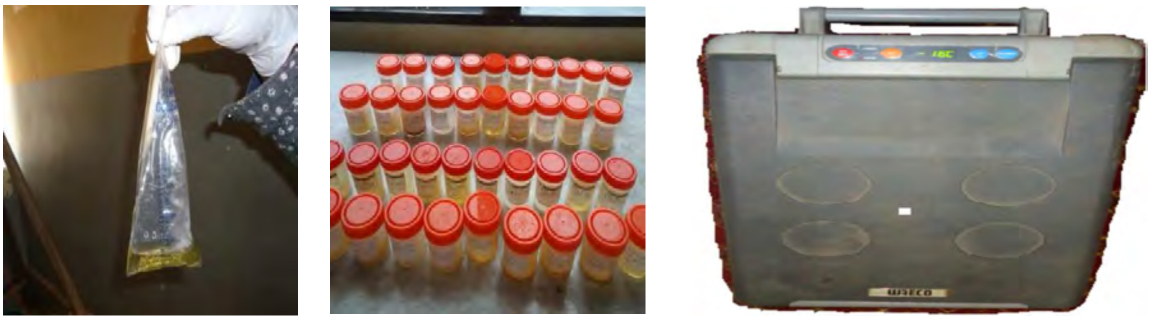

Figure 6.2 Urine sample collection, storage and transportation from field to Center for Food Science and Nutrition, AAU

6.4.6 Urinary aflatoxin analysis

6.4.6.1 Reagents and chemicals

AFB1, AFB2, AFG1, AFG2 and AFM1 (1 mg) were purchased in powder form at Sigma-Aldrich (Diegem, België). They were reconstituted in 1 mL of methanol and stored at -18°C. From the individual stock solutions, standard mixtures of 25 ng/mL and 2.5 ng/mL were prepared in methanol, stored at -18°C and renewed monthly. Starting from these mixtures, a calibration curve with five points, excluding blank, was made each time an analysis was started. Purified water was obtained from a Milli-Q® water system (Millipore, Brussels, Belgium). Methanol (LC-MS grade) and glacial acetic acid (LC-MS grade) were purchased from BioSolve (Valkenswaard, The Netherlands). Ammonium acetate was supplied by Merck (Darmstadt, Germany).

6.4.6.2 LC-MS/MS methodology

Chromatographic separation was performed on a Waters Acquity UPLC® H-Class (Milford, MA, USA) equipped with a flow through needle sample manager. The analytical column used was a Waters Acquity UPLC® HSS T3 (1.8 µm, 2.1 mm × 100 mm) column kept at 40 °C. This column was preceded with a Waters Acquity UPLC® BEH C18 Van Guard pre-column (1.7 µm, 2.1 mm x 5 mm). Two mobile phases were used: Eluent A was ultrapure water with 5 mM ammonium acetate and 0.1% acetic acid. Eluent B consisted of methanol containing 0.1% acetic acid. The flow rate used was set at 400 µL/min. A gradient elution was applied which started at 50% mobile phase B for 1 min, followed by a linear increase from 50% to 75% mobile phase B during 2 min, after which the column was equilibrated at initial conditions, resulting in a total run time of 5 min. The auto sampler temperature was set at 10°C.

A Xevo TQ-S (Waters, Manchester, UK) equipped with an electrospray ionization (ESI) source, was used as detector. In order to achieve optimal sensitivity and selectivity of the MS conditions, data acquisition was performed applying multiple reaction monitoring (MRM) with an optimized dwell time (0.019 s). For each target analyte, two precursors to fragment ion transitions were determined. In accordance with Commission Decision (EC) No. 657/2002, laying down the performance criteria of analytical methods, the use of two transitions allows determination of the ratio between these two transitions (relative ion intensity). This ratio, together with the retention time and signal-to-noise ratio (S/N), allows the confirmation of the identity of the detected compound. The most abundant fragment ion was used for quantification, the second one for confirmation purposes.

MS/MS instrumental parameters were optimized via direct infusion (flow rate of 10 µL/min) of a tune solution (10 ng/mL of each analyte, dissolved in methanol/ultrapure water (50/50; v/v) containing 0.1% acetic acid). The mass spectrometer was operated in the positive (ESI+)

electrospray ionization mode. The source temperature was set at 150°C while the capillary desolvation heater was set at 450°C. The capillary voltage used was 0.5 kV, while the drying gas was nitrogen at a flow rate of 1000 L/h. The MRM transitions, optimum cone voltages and collision energies selected for each transition are given in Table 6.1, as well as the indicative retention times on the column. MassLynx® 4.1. and TargetLynx® 4.1 software (Micromass, Manchester, UK) were used for data acquisition and processing.

Table 6.1 Optimized LC-MS/MS instrumental parameters for all analytes

Analyte	Ionisation mode	Precursor ion (m/z)	Fragment ion (m/z)	Cone voltage (V)	Collision energy (eV)	Retention time (min)
AFB_1	$[M+H]^+$	313	241*	60	35	1.88
			285		19	
AFB_2	$[M+H]^+$	315	259	60	28	1.83
			287*		25	
AFG_1	$[M+H]^+$	329	200	60	37	1.62
			243*		25	
AFG_2	$[M+H]^+$	331	189	60	28	1.42
			245*		38	
AFM_1	$[M+H]^+$	329	229	60	25	1.42
			273*		25	

* Most abundant fragment ion and used for quantification.

6.4.6.3 Sample preparation methodology

Prior to analysis, urine samples were thawed at room temperature and vigorously vortexed. Five hundred microliter (500 µL) of the samples were subjected to ultracentrifugation for 5 min at 10,000 × g using Ultrafree® MC centrifugal devices (Millipore, Bedford, MA, USA) to remove the majority of solid material. Ten microliters (10 µL) of the supernatant were injected into the chromatographic system.

6.4.6.4 Method validation

Due to the unavailability of certified reference material, optimization and validation of the multi-mycotoxin analytical method for human urine was performed using spiked blank samples. The LC-MS/MS method was successfully validated for the five aflatoxins in human urine according to Commission Decision (EC) No. 2002/657 and Commission Regulation (EC) No. 401/2006 including following validation parameters: specificity, linearity, matrix effect, apparent recovery, repeatability (intraday precision; RSD_r) and reproducibility (intermediate precision; RSD_R). Limit of detection (LOD) and limit of quantification (LOQ) were assessed according to international conference on harmonization (ICH) guidelines. All analytes were quantified using matrix-matched calibration curve and all validation parameters were calculated using the peak area of the analyte.

i. Specificity and matrix effect:

Specificity concerns the ability of the analytical method to distinguish between the analyte being measured and other substances or interferences. In order to determine the specificity, 10 representative blank samples and 3 blank samples fortified with other major mycotoxins were subjected to analysis, revealing no interfering peaks ($S/N \geq 3$) in the 2.5% margin of the retention time for all target analytes.

Matrix effects result from co-eluting residual matrix components affecting (suppression or enhancement) the ionization efficiency of target analytes. In extreme cases, these matrix effects can result in complete suppression of the analytical signal. Since matrix effects may exert a negative effect on important method performance parameters and subsequently lead to erroneous quantitative results, they have to be tested for and evaluated during method development and validation. Matrix effects for each analyte were assessed whereby calibration curves in neat solvent were constructed by plotting the signal intensity versus the analyte concentration. Likewise, signal intensities obtained from spiking the samples were plotted against the actual spiking levels. Subsequently, using the slopes of the resulting functions, the signal suppression/enhancement (SSE) due to matrix effects were calculated. An ion suppression was observed for aflatoxins whereby SSE was between 54% and 76% (Table 6.2). Furthermore, comparison by visual inspection of the slopes confirmed the presence of matrix effects, and indicated the need to use matrix specific curves for quantification purposes (Figure 6.3).

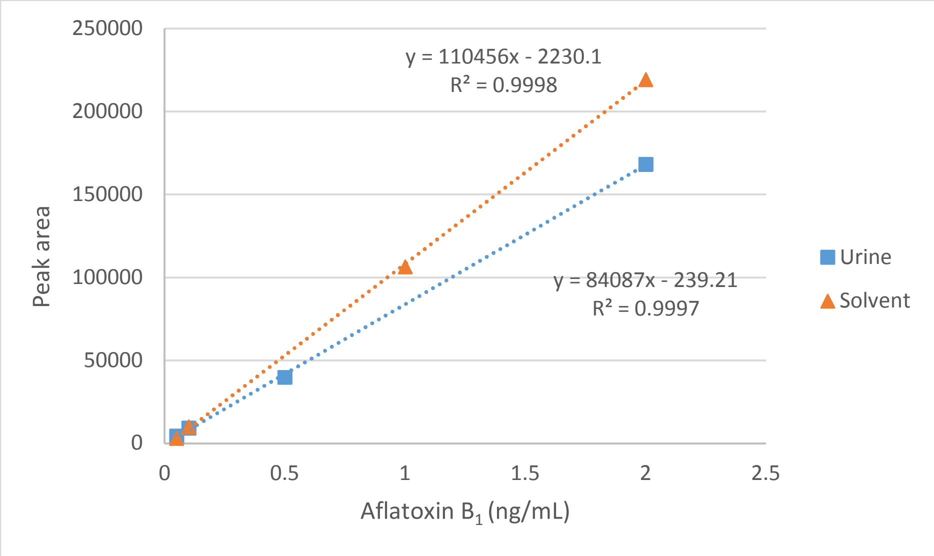

Figure 6.3 Comparison of the slopes of calibration curves of aflatoxin B1 in urine and standard solution, revealing the presence of varying (matrix dependent) signal suppression.

ii. Linearity, LOD and LOQ:

Linearity was evaluated using matrix matched calibration (MMC) curves, by spiking blank samples (urine) at five concentration levels (0.05, 0.1, 0.5, 1, 2 ng/mL) in triplicate. In addition to the commonly reported regression coefficients (R^2), with 0.9986 being the lowest observed value, a lack-of-fit test (SPSS) was also performed to assess the adequacy of the linear model. The linearity of the peak areas for all analytes was excellent in the applied range, with p-values greater than 0.05, which demonstrated no lack of fit and thus good adequacy of the models in predicting linearity.

Furthermore, the condition of equal variances, termed homoscedasticity (homogeneity of variance), was tested in the applied linear regression analysis. When the assumption of homoscedasticity is not met for analytical data, an effective way to counteract the greater influence of the greater concentrations on the fitted regression line is to use weighted least squares linear regression. By plotting residuals, the weighing factor 1/x provided the most adequate approximation of variance for all analytes. Additionally, weighing factor 1/x drastically improved the accuracy at the lowest concentration level of the calibration curve.

LOD and LOQ were determined using MMC curves (in triplicate), by spiking blank samples at 5 concentrations levels (0.05-2 ng/mL) (Table 6.2). LOD and LOQ for the different components was calculated by TargetLynx® 4.1 defining a minimum S/N of 3 and 10, respectively. Although no regulatory limits have been set for aflatoxins in human urine, the developed UPLC-MS/MS methodology allowed for the determination of all target analytes at low levels (ng/mL).

Table 6.2 Linear range, limit of detection (LOD), limit of quantification (LOQ) and signal suppression/enhancement (SSE) for aflatoxins in human urine

Analytes	**Linear range (ng/mL)**	**LOD (ng/mL)**	**LOQ (ng/mL)**	**SSE (%)**
Aflatoxin B_1	0.05-2	0.025	0.05	76
Aflatoxin B_2	0.05-2	0.025	0.05	67
Aflatoxin G_1	0.05-2	0.025	0.05	65
Aflatoxin G_2	0.05-2	0.025	0.05	62
Aflatoxin M_1	0.05-2	0.025	0.05	54

iii. Apparent recovery, repeatability and reproducibility:

Apparent recovery, repeatability and intermediate precision were all calculated from a combined sample plan, which comprised of the analysis of blank samples spiked in triplicate with the different mycotoxins at concentration levels of 0.05, 0.1, 0.5, 1 and 2 ng/mL on three consecutive days (Table 6.3). The apparent recovery was determined using MMC curves (five concentration levels, 0.05-2 ng/mL). The measured concentration of the spiked blank samples was determined by plotting the observed signal (expressed as the peak area) into the corresponding MMC curves. IUPAC defines the apparent recovery as the ratio of the predicted value obtained from the MMC curves divided by the actual/theoretical value. The apparent recovery at the five concentration levels ranged from 90.1% till 119.6% for all analytes. These results are in good agreement with the guideline ranges (80–120%) imposed by Commission Decision No. (EC) 2002/657.

Repeatability (RSD_r) and intermediate precision (RSD_R) relative standard deviations were calculated using one-way analysis of variance (ANOVA). RSD_r and RSD_R, ranged from 1% to 13% and 4% to 34%, respectively. According to an in-house developed standard operating procedure on analytical method validation, RSD_r and RSD_R values for concentrations lower than 100 ng/mL should be lower than 20% and 25%, respectively.

Table 6.3 Apparent recovery, repeatability (RSD_r) and reproducibility (RSD_R) for aflatoxins in human urine at five concentration levels

Concentration (ng/mL)	AFB_1			AFB_2			AFG_1			AFG_2			AFM_1		
	Apparent recovery (%)	RSD_r (%)	RSD_R (%)	Apparent recovery (%)	RSD_r (%)	RSD_R (%)	Apparent recovery (%)	RSD_r (%)	RSD_R (%)	Apparent recovery (%)	RSD_r (%)	RSD_R (%)	Apparent recovery (%)	RSD_r (%)	RSD_R (%)
0.05	94.44	5	12	111.33	9	11	102.22	9	12	119.56	13	17	114.89	6	13
0.1	107.89	3	4	110.67	5	4	107.22	3	11	108.78	7	13	118.67	5	17
0.5	104.89	1	17	105.60	1	19	109.00	3	21	109.44	4	19	115.16	5	33
1	92.90	4	24	92.73	2	23	91.14	3	22	90.08	5	30	95.11	4	34
2	105.39	3	9	103.43	4	6	104.89	6	11	101.56	8	18	108.34	11	24

6.4.7 Data analysis

Data from the FFQ were processed using SPSS statistical software version 20. The anthropometric data were processed using ENA for SMART 2011 software (Emergency Nutrition Assessment: 2003-2015 Michael Golden/John Seaman/Juergen Erhardt). The levels of urinary aflatoxin among regions and the associations between urinary aflatoxin and socio-demographic factors were processed using paired sample t-test. The correlations between urinary aflatoxin occurrences and anthropometric measurements was processed using Pearson correlation. p-value < 0.05 was considered as statistically significant.

6.5 Results and discussion

6.5.1 Descriptive data on the characteristics and staple foods of the young children

The socio demographic information for the 200 children is presented in Table 6.4. With respect to age, except in three Districts (Baso Liben, Awabel and Sekela) of Amhara region, the numbers of children in the two age categories (1-2 and above 2-4 years) are equal. Overall, 48% (96) of the children were between ages of one to two years while 52% (104) were between two and four. With respect to gender, except one District (Sekela) of Amhara region, the numbers of male and female children are almost comparable. Of the 200 children included in the study, 49.5% (99) were boyss while 50.5% (101) were girls.

With respect to the weaning status, the children were classified in two categories as: partially breast feed or fully weaned (no breast milk). Among the 200 children, 62.5% (125) were partially breast feed while 37.5% (75) were fully weaned. The percentage distribution of the partially breast feed and fully weaned children in each District are indicated Table 6.4. In most Districts, children greater than two years were either partially breast feed or fully weaned.

Table 6.4 The socio demographic information for the young children across the two regions

Region	District	Characteristics				
		Number of children per District	**Age in years (1-2: >2-4)**	**Sex (male: female)**	**Number of partially breast feed children (%)**	**Number of fully weaned children (%)**
	Baso Liben	20	8:12	7:13	14 (70%)	6 (30%)
	Awabel	20	9:11	7:13	17 (85%)	3 (15%)
Amhara	Machakel	20	10:10	9:11	18 (90%)	2 (10%)
	Burie	20	10:10	11:9	18 (90%)	2 (10%)
	Sekela	20	9:11	14:6	13 (65%)	7 (35%)
	Endemekhoni	20	10:10	10:10	9 (45%)	11 (55%)
	Samre Saharti	20	10:10	10:10	7 (35%)	13 (65%)
Tigray	Kilte Awlalo	20	10:10	10:10	9 (45%)	11 (55%)
	Adwa	20	10:10	10:10	11(55%)	9 (45%)
	Atsbi Womberta	20	10:10	11:9	9 (45%)	11 (55%)

Each mother/caregiver was asked to indicate the types of food they fed to their child in the week prior to the sample collection and the frequency of consumption through an FFQ. Based on this seven days FFQ, the children in both regions were generally consumed starchy stable foods/food products based on teff (in the forms of "Enjera"- pan cake) (35% (70)) two to three times per day, wheat (in the form of bread) (26.5% (53)) one time per day, broad bean and pea (in the forms of "Shiro wot"-stew) one time per day. Usually "Enjera" is eaten with or accompanied by "Shiro wot" made from broad bean and pea blend. In addition, very few children, 11% (22) consumed porridge made from a combination of maize, wheat and broad bean two to three times per week. Very few children, 9% (18) and 9.5% (19) also consumed wheat and maize respectively in the forms of "Kollo" (roasted wheat or maize) two to three times per week. In addition to starchy stable foods, some children (18.5% (37)) consumed milk (one cups of milk) two to three times per week. While, 26% (52) consumed egg (one egg) two to three times per week. The types and frequency of consumption of food across the two regions studied are depicted in Table 6.5.

Table 6.5 Types of food eaten by the young children in the study areas and frequency of consumption

Food category	Number of intakes in days per week	Regions		Total
		Amhara	Tigray	
1. "**Enjera**"- pan cake, made from:				
✓ Teff	2-3	16 (16%)	54 (54%)	70 (35%)
✓ Maize	2-3	5 (5%)	0 (0%)	5 (2.5%)
✓ Teff and maize blend	2-3	51 (51%)	3 (3%)	54 (27%)
✓ Barley	2-3	0 (0%)	32 (32%)	32 (16%)
2. **Bread** made from:				
✓ Wheat	2-3	23 (23%)	48 (48%)	71 (35.5%)
✓ Wheat and maize blend	2-3	10 (10%)	0 (0%)	10 (5%)
3. "**Shiro wot**"- stew, made from:				
✓ Broad bean	2-3	23 (23%)	14 (14%)	37 (18.5%)
✓ Pea	2-3	27 (27%)	18 (18%)	45 (22.5%)
✓ Grass pea	2-3	1 (1%)	15 (15%)	16 (8%)
4. **Porridge** made from:				
✓ Maize, wheat, teff, broad bean and pea blend	2-3	21 (21%)	0 (0%)	21 (10.5%)
✓ Wheat, maize and pea blend	2-3	1 (1%)	12 (12%)	13 (6.5%)
✓ Wheat, maize and broad bean blend	2-3	9 (9%)	8 (8%)	17 (8.5%)
✓ Wheat, teff and broad bean blend	2-3	3 (3%)	19 (19%)	22 (11%)
5. "**Kollo**"- roasted, made from:				
✓ Maize	1	24 (24%)	3 (3%)	27 (13.5%)
✓ Wheat	1	6 (6%)	14 (14%)	20 (10%)
6. **Milk**	2-3	14 (14%)	23 (23%)	37 (18.5%)
7. **Egg**	2-3	21 (21%)	31 (31%)	52 (26%)
8. **Beef**	2-3	40 (40%)	31 (31%)	71 (35.5%)
9. **Peanut butter**	2-3	1 (1%)	0 (0%)	1 (0.5%)

6.5.2 The anthropometric characteristics of the young children

The prevalence of malnutrition in the young children recruited for this study is depicted in Table 6.6. There was a strong prevalence of stunted growth (low HAZ), low underweight (WAZ) but very low wasting (WHZ). Of the 200 children, 26.5% (53) were moderately stunted (<-2SD) while 18.5% (37) were severely stunted (<-3SD) according to the World Health Organization 2006 criteria (WHO, 2006). With respect to gender, males were more stunted than females. The percentage distribution of severely and moderately stunted boys and girls respectively were 20.2% (20), 16.8% (17) and 29.3% (29), 23.8% (24). Among the two regions, Tigray had the highest percentage of severely (22% (22)) and moderately (28% (28)) stunted children. Our result is consistent with the previous Ethiopian mini demographic and health survey (CSA, 2014) reported that the percentage of stunted children in Tigray (44%) is higher than in Amhara (42%) region. The mean values for each of the three growth parameters WHZ, WAZ and HAZ across the regions were below zero (mean = −0.09, −1.00, −1.74, respectively) with particularly low values for HAZ and WAZ. The distributions of WHZ, WAZ, and HAZ (all children <-2SD) in children across the two regions are indicated in Figure 6.4.

Table 6.6 Prevalence of malnutrition in children in two regions of Ethiopia, based on the World Health Organization guidelines

Criteria	**Total number of children**	**Prevalence rate**		**Total**
		Moderately malnourished (<-2SD)	**Severely malnourished (<-3SD)**	
WHZ-Wasting	200	(1) 0.5%	(1) 0.5%	2 (1%)
WAZ-Under weight	200	(33) 16.5%	(1) 0.5%	34 (17%)
HAZ-Stunting	200	(53) 26.5%	(37) 18.5%	90 (45%)

WHZ-weight for height Z-score; WAZ- weight for age Z-score; and HAZ- height for age Z-score.

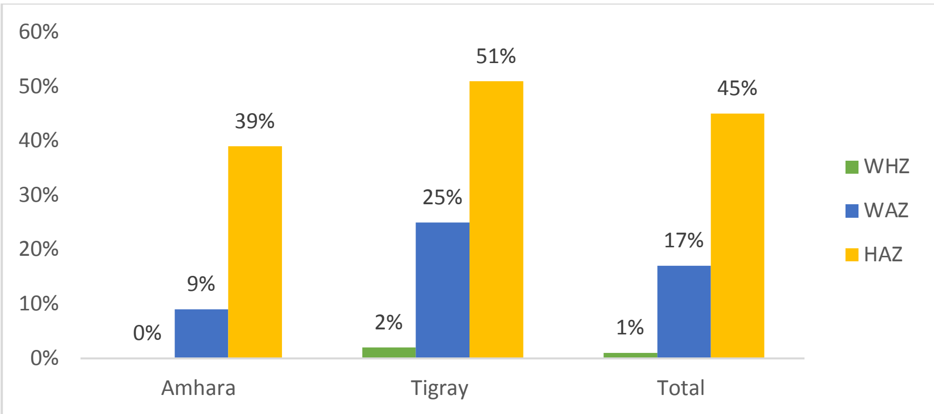

Figure 6.4 The distributions of both moderately and severely malnourished children across the two regions in Ethiopia

6.5.3 Distribution of aflatoxin contamination and co-occurrence rate in the urine samples

The urine samples from the young children were analyzed for a total of five aflatoxins; AFB1, AFB2, AFG1, AFG2 and AFM1. One or more aflatoxins were detected in 17% (34/200) urine samples collected from the two regions. In terms of occurrence, AFM1 was the most frequently detected (7% (14/200)), followed by AFB2 (4.5% (9/200)), and AFG2 (3% (6/200)), while AFG1 (2.5% (5/200)) was less detected. However, AFB1 was not detected in any of the urine samples. In terms of co-occurrence, two aflatoxins (AFB2 & AFG2; AFB2 & AFG1 and AFB2 & AFG1) co-occurred in three different samples, while three aflatoxins (AFB2, AFG1 & AFM1) were co-occurred in one sample. Table 6.7 shows the occurrence and level of urinary aflatoxin concentration from the urine samples collected across all the regions.

Table 6.7 Occurrence of aflatoxins in 200 urine samples from young children collected from two regions in Ethiopia

Analytes	Number of positive samples (%)	Urinary aflatoxin concentration: mean (range) (ng/mL)
AFB1	0 (0%)	<LOD
AFB2	9 (4.5%)	0.047 (<LOQ-0.063)
AFG1	5 (2.5%)	0.061 (0.054-0.065)
AFG2	6 (3%)	0.068 (0.066-0.070)
AFM1	14 (7%)	0.064 (0.063-0.070)
Total	34 (17%)	-

AFB1: Aflatoxin B1, AFB2: Aflatoxin B2, AFG1: Aflatoxin G1, AFG2: Aflatoxin G2, AFM1: Aflatoxin M1. Limit of detection (LOD) = 0.025 ng/mL and Limit of quantification (LOQ) = 0.05 ng/mL for each aflatoxin. The means are the average of the positive samples.

Urinary aflatoxin biomarkers are useful measures of the recent ingestion of aflatoxins. More specifically, urinary AFM1 has been well established as a biomarker of exposure (Ezekiel *et al*., 2014; Mykkanen *et al*., 2005; Polychronaki *et al*., 2008). In this study, AFM1was the most frequently detected urinary aflatoxin metabolite with a mean concentration of 0.064 ng/mL. The presence of AFM1 in the urine samples clearly indicates the contamination of the children's diet with AFB1 (Schwartzbord *et al*., 2016; Shephard *et al*., 2013). Even though there are differences in detection limits and other factors, our result is lower than a previous study by Ediage *et al*. (2013) who reported the mean and range (0.33; 0.06-4.7 ng/mL) of AFM1 in urine samples collected from young children in Cameroon. A similar study by Ezkiel *et al*. (2014) in Nigeria also reported higher AFM1 levels (mean; 0.3 ng/mL) in urine samples collected from children. However, our result is higher than a previous study by Polychronaki *et al*. (2008) who reported the mean and range (0.006; 0.005-0.006 ng/mL) of AFM1 in urine samples collected from Egyptian children. Another study by Hatem *et al*. (2005) in Egypt also reported lower AFM1 levels (mean; range) (0.023; 0.01-0.03 ng/mL) and (0.055; 0.04-0.07 ng/mL) in urine samples of children with kwashiorkor and marasmus respectively.

AFB2 was the second most frequently observed (4.5%) urinary aflatoxin in this study with a mean concentration of 0.047ng/mL. This might be due to the contamination of the children's food with AFB2 and there is also the possibility of the hepatic conversion of AFB1 into AFB2 (Polychronaki *et al*., 2008). Although the levels of AFB2 in the urine samples in our study is minimum, but it is higher than a previous study by Polychronaki *et al*. (2008) who reported AFB2 (mean and range: 0.0014; 0.0008-0.0022 ng/mL) and (mean and range: 0.0057; 0.0006-0.043 ng/mL) in urine samples collected from Guinean and Egyptian children respectively. Another study by Hatem *et al*. (2005) in Egypt also reported lower levels of AFB2 (mean; 0.027 ng/mL) and (mean; 0.02 ng/mL) in urine samples of children with kwashiorkor and marasmus respectively.

Even though, the frequency of occurrence was very small, AFG2 (3%) and AFG1 (2.5%) were also observed in urine samples in this study with a mean concentration of 0.068 ng/mL and 0.061 ng/ mL respectively. A study by Polychronaki *et al*. (2008) reported 0.002 ng/mL and 0.019 ng/mL mean AFG2 in urine samples collected from Egyptian and Guinean children respectively, although the mean concentration in our study was a bite higher. The levels of AFG1 detected in our study is minimum, but still higher than a previous study in Egypt by Hatem *et al*. (2005) who reported AFG1 (mean; 0.048 ng/mL and 0.036 ng/mL) levels in urine samples of the children with kwashiorkor and marasmus respectively. However, a study by Polychronaki *et al*. (2008) reported higher AFG1 levels (mean; 0.077 ng/ml and 0.709 ng/ml) in urine samples collected from Egyptian and Guinean children respectively.

However, in our study AFB1 was not detected in any of the urine samples. This might be due to the biotransformation of AFB1 into AFM1 and AFQ1 with the help of cytochrome P450 enzymes (Mykkanen *et al*., 2005; Polychronaki *et al*., 2008). But previous studies (Hatem *et al*. 2005; Polychronaki *et al*., 2008) detected AFB1 in urine samples collected from young children. This might be due to differences in age, detection limits and analytical performances of the methods.

When we compare the frequency of aflatoxin occurrence in urine among the two regions, the exposure in Tigray (18% (18/100)) was larger than Amhara (16% (16/100)) region. Besides, there was a significant difference between the mean concentrations of total urinary aflatoxins (AFB1 +AFB2 + AFG1 +AFG2 +AFM1) ($p < 0.05$) among the two regions. The incidence and levels of urinary aflatoxins in samples from the two regions are presented in Table 6.8.

Table 6.8 Incidence and levels of urinary aflatoxins in urine samples from children collected from two regions in Ethiopia

Region	District	Number of samples analyzed	Number of positive samples (%)	Mean urinary aflatoxin concentration in ng/mL (number of samples)				
				AFB1	**AFB2**	**AFG1**	**AFG2**	**AFM1**
	Baso Liben	20	4 (20%)	<LOD	<LOD	0.065 (2)	0.067 (1)	0.063 (1)
Amhara	Awabel	20	1 (5%)	<LOD	<LOD	<LOD	<LOD	0.063
	Machakel	20	1 (5%)	<LOD	<LOD	<LOD	<LOD	0.064
	*Burie	20	7 (35%)	<LOD	0.049(2)	<LOD	0.068 (2)	0.064 (3)
	Sekela	20	3 (15%)	<LOD	<LOD	<LOD	0.068 (1)	0.067 (2)
	Endemekhoni	20	4 (20%)	<LOD	<LOD	<LOD	0.066 (1)	0.063 (3)
Tigray	*Atsbi Womberta	20	5 (25%)	<LOD	0.062(2)	0.065 (1)	<LOD	0.063 (2)
	Kilte Awlalo	20	0 (0%)	<LOD	<LOD	<LOD	<LOD	<LOD
	*Samre Saharti	20	2 (10%)	<LOD	0.035(1)	0.054 (1)	<LOD	<LOD
	**Adwa	20	7 (35%)	<LOD	0.041(4)	0.055 (1)	0.07 (1)	0.064 (1)
	Amhara region mean of urinary aflatoxin (ng/mL) ±SD			0.063±0.008[a]				
	Tigray region mean of urinary aflatoxin (ng/mL) ±SD			0.055±0.013[b]				

AFB1: Aflatoxin B1, AFB2: Aflatoxin B2, AFG1: Aflatoxin G1, AFG2: Aflatoxin G2, AFM1: Aflatoxin M1. Limit of detection (LOD) = 0.025 ng/mL and Limit of quantification (LOQ) = 0.05 ng/mL for each aflatoxin. The means are the average of the positive samples. * Two aflatoxins (AFB2 & AFG2, AFB2 & AFG1, AFB2 & AFG1) co-occurred in three different samples and ** Three aflatoxins (AFB2, AFG1 & AFM1) co-occurred in one sample.

6.5.4 Correlation between the different urinary aflatoxin concentrations and socio-demographic factors and anthropometric characteristics

A pairwise comparison of the data was performed in order to exploit the possible correlation/associations between the urinary aflatoxin concentrations and some socio-demographic factors. Table 6.9 shows the mean urinary aflatoxin concentrations of each analyte for the different socio-demographic categories.

Table 6.9 Distribution of urinary aflatoxin contamination by age, gender, region and weaning status

Variable/factors	**Number of children**	**Number of positive samples**	**Aflatoxin contamination level: mean (ng/mL)**				
			AFB1	**AFB2**	**AFG1**	**AFG2**	**AFM1**
Total	200	34	<LOD	0.047	0.061	0.068	0.064
Age group							
1-2 years	96	16	<LOD	0.055	<LOD	0.068	0.065
>2-4 years	104	18	<LOD	0.042	0.061	0.068	0.063
Gender							
Male	99	16	<LOD	0.055	0.062	0.068	0.066
Female	101	18	<LOD	0.041	0.06	0.068	0.063
Region							
Amhara	100	16	<LOD	0.049	0.065	0.068	0.064
Tigray	100	18	<LOD	0.062	0.058	0.068	0.063
Weaning status							
Partially breast feed	125	20	<LOD	0.062	0.065	0.068	0.064
Fully weaned	75	14	<LOD	0.044	0.06	0.068	0.064

AFB1: Aflatoxin B1, AFB2: Aflatoxin B2, AFG1: Aflatoxin G1, AFG2: Aflatoxin G2, AFM1: Aflatoxin M1. The limit of detection (LOD) = 0.025 ng/mL and Limit of quantification (LOQ) = 0.05 ng/mL for each aflatoxin. The means are the average of the positive samples.

The mean urinary aflatoxin concentrations measured from the young children in this study differed non-significantly ($p > 0.05$) among the two age groups. However, AFG1 was not detected in the urine samples collected from children between one and two years, while AFG1 was detected in urine from children between two and four years old. This difference might be due to differences in the amount of food intake. Similarly, there is no significant difference ($p > 0.05$) in the mean urinary aflatoxin concentration between male and female children. Our result is in agreement with a previous study performed in Cameroon by Ediage *et al*. (2013) who reported that the mean urinary AFM1 concentrations among male and female children were non-significantly different. Another study in Egypt by Hatem *et al*. (2005) reported that no significant difference between males and females was found in the urinary levels of aflatoxins in children with marasmus or kwashiorkor.

With respect to region, Tigray (18%) was greater than Amhara (16%) in terms of frequency of observed urinary aflatoxin. Besides, there was a significant difference between the mean concentrations of total urinary aflatoxins among the two regions. However, the mean concentrations of each types of urinary aflatoxin were statistically the same across the two regions ($p > 0.05$). Therefore, this result indicated that the risks of mold and aflatoxin contamination of the children's diet during the sample collection across the two regions might be the same.

With respect to weaning status, fully weaned children (18.6%) had more frequently observed urinary aflatoxin levels than partially breast feed children (16%). However, no significant difference ($p > 0.05$) was found in the mean concentrations of all of the urinary aflatoxin analytes among the partially breast feed children and fully weaned children. In contrast to our results, a study in Cameroon by Ediage *et al*. (2013) reported that the mean AFM1 concentration detected from the fully weaned children (0.282 ng/mL) was significantly different from those who were partially breast fed (1.43 ng/ml).

Correlation (Pearson correlation) was also performed to investigate the possible associations between the respective urinary aflatoxin occurrences and the different anthropometric measurements. No associations were observed between the presence of urinary aflatoxin and being stunted (r: -0.038 and p= 0.598), underweight (r: -0.035 and p=0.623), and wasted (r: -0.004 and p= 0.952) ($p > 0.05$). However, our result is consistent with the previous study in Cameroon by Ediage *et al*. (2013) who reported that no association was observed between the different malnutrition categories (stunted, wasting and underweight) and the mycotoxin concentrations detected in the urine of young children.

6.6 Conclusion

For the first time in Ethiopia, this study assessed aflatoxin exposure among young children using urinary biomarkers. The biomarker analysis showed a clear exposure of the young children to aflatoxins. AFM1 was the most frequently detected urinary aflatoxin metabolite but AFB1 was not detected in any of the urine samples. Therefore, this study could be considered as a pilot study to raise awareness to the public about the potential health consequences of aflatoxin among young children.

CHAPTER 7. GENERAL CONCLUSION AND RECOMMENDATION

7.1 Conclusion

The interviewed mothers/caregivers in this study across four regions of Ethiopia were reportedly aware of a range of key practices to prevent the risks of mold and aflatoxin contamination farm-to-table. Their reported knowledge on issues such as appropriate ploughing, crop rotation, and debris removal should contribute to the prevention of mold growth. However, the threshing methods (trampling by hoofed animals), common storage methods of 'Gota', 'Gotera' and underground pit, followed by households and grain banks storage in plastic containers are likely contributing to the mold growth found in this study. Further, the reported practices of consuming moldy cereals and legumes in times of scarcity and of feeding these to animals that could later be consumed by the family are additional risks of contamination, particularly among young children. Hence, although agricultural practices were positive, respondents did not appear to understand the potentially toxic effects of directly or indirectly consuming contaminated foods or animals that had consumed these foods on the health of humans and animals.

The detection of *C. sakazakii*, coliforms and *E. coli* in the CFs and their ingredients might have a health risk to the young children that must be addressed through improved conditions of sanitation and hygienic practices. The identification of more contamination in CFs stored for one month implies poor hygienic practices in both households and grain banks. Aflatoxins were detected in nearly all samples, but very few samples exceeded a toxic level (10 µg/kg). Although, aflatoxin levels were considered safe for consumption in most samples, more efforts should be implemented to reduce the contamination level, particularly as these CFs are intended for consumption by young children.

Young children (< 5 years of age) raised in Amhara and Tigray regional states of Ethiopia are exposed to aflatoxins during infancy as revealed by biomarker analysis. As yet, there is no study about the health effects of aflatoxins in children in Ethiopia, therefore, this study could be considered as a pilot study to raise awareness to the public about the potential health consequences of aflatoxin among young children.

7.2 Recommendation

In view of the results of this study, the following recommendations are made:

- ✓ The common storage methods for cereals and legumes used were ‘Gota’ ‘Gotera’ and underground pit, all of which are susceptible to mold spoilage and aflatoxin contamination. Therefore, introduction of modern storage structures is recommended to prevent the risks of aflatoxin contamination.
- ✓ Awareness creation to the public (mothers/caregivers, health extension workers, agricultural development agents and other stakeholders) about the methods used to prevent and minimize the risks of mold spoilage and aflatoxin contamination in cereals and legumes, human and animal health effects and economic impacts of aflatoxins.
- ✓ More research is needed to determine conditions that influence the survival and growth of *C. sakazakii* in locally prepared CFs.
- ✓ Regulatory limits for AFB1 and total aflatoxins need to be devised in locally as well as commercially prepared cereal based CFs in Ethiopia in order to protect the young children from the risk of aflatoxins exposure.
- ✓ Further studies are needed to assess multiple occurrences of mycotoxins in the CFs and their ingredients.
- ✓ More research is needed for the introduction of mold resistant seed variety. Besides, bio-competitive non-toxigenic fungal strains such as Aflasafe^{TM} to exclude toxigenic fungi need to be introduced in Ethiopia.
- ✓ Further studies to investigate the association between aflatoxin exposure and child growth in Ethiopia.
- ✓ Further studies about the incidence and levels of AFM1 in breast milk.
- ✓ Further studies about the impact of HACCP based SOPs after implementation.

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ANNEXES

ANNEXE A:

Annex A1. Information and Consent

Dear respondent,

Good morning/Good afternoon. Thank you for your interest to talk with me today. I am ________________________________ who is a member of a team from Addis Ababa University conducting a study to assess the safety of complementary foods prepared from locally available ingredients in your locality. The purpose of my visit today is to take information from you on the aforementioned issue. If you are willing to participate in the study, I will ask you few questions for 20-30 minutes. Your name will not be written on this form and will never be used in connection with any of your information. You do not have to answer any question that you are not comfortable with, and you may end this task any time you want to. However, your honest answers to these questions will help us in better understanding of the safety of complementary foods and its ingredients in your locality, and will eventually help in designing and implementing appropriate interventions to alleviate related problems.
Hence, we greatly appreciate your participation in the study.

Are you willing to participate in the study? Yes ☐ No ☐

Signature of the data collector

Name: ________________________________ Signature: ________________ Date: _________

Annex A2: Survey Questionnaire for Assessing the Safety of Complementary Foods Produced at Community Level Using Locally Available Ingredients in Ethiopia

1. Questionnaire Code: _______________ Location (Region): ___________________________
2. District (Kebele): _________________ Woreda: _______________________________________
3. Set up: 1. Rural 2. Semi-urban
4. Date of youngest child's birth (months): _______________ (between 6 to 24 months).
5. Mother's age in years: ____
6. Education level of mother or care giver:
   1. Illiterate
   2. Can Read and/or Write (informal education)
   3. Grade 1-4
   4. Grade 5-8
   5. Grade 9-12
   6. Tertiary education
7. Language spoken by the family:
   1. Tigrigna
   2. Amharic
   3. Afan Oromo
   4. Guragena
   5. Hadiya
   6. Sidma
   999-Missing

<u>1: Pre-harvest knowledge and practice information</u>

1. Do you plow the land after growing maize (other previous crops) before growing the next crop?
   1. Yes
   2. No
   3. Not applicable
   999-missing
2. If your answer for Q.1 is yes, how many times do you plow?
   1. Only once
   2. Two times
   3. Three times
   4. More than three times
   5. Not applicable
   999. Missing
3. Do you use a crop rotation schedule to avoid planting the same commodity in a field?
   1. Yes
   2. No
   3. NA
   999. Missing
4. Do you have the practice of removing old seed heads, stalks, and other debris that may have served, or may potentially serve as substrates for the growth of aflatoxin-producing fungi?
   1. Yes
   2. No
   3. NA
   999. Missing
5. Did you use seed varieties for cereals and legumes developed for resistance to seed infecting fungi and insect pests?
   1. Yes
   2. No
   3. NA
   999. Missing
6. Do you know that some climatic conditions (temperature, rain or humidity, drought, etc.) are conducive for the proliferation of poison forming molds in crops?
   1. Yes
   2. No
   3. NA
   999. Missing
7. If your answer for Q.6 is yes, which climatic factor mostly cause for the formation of poison forming molds?

1) Hot temperature
2) Rain
3) Drought
4) Humid seasons
5) 1 &2
6) 1, 2 & 4
7) 1, 2, 3 & 4
8) NA
999.Missing

8. Which crop type is more susceptible to mold spoilage?
   1) Cereals
   2) Legumes
   3) NA
   999. Missing
9. If your answer for Q.8 is Cereals, which cereals are more susceptible?
   1) Maize
   2) Sorghum
   3) Wheat
   4) Barley
   5) Oat
   6) Teff
   7) Finger millet
   8) Engedo (?)
   9) NA
   999. Missing
10. Have you ever used insecticides and fungicides to minimize insect damage and fungal infection when the cereals and legumes are growing in the field?
    1. Yes
    2. No
    3. NA
    999. Missing
11. If your answer for Q.10 is yes, when do you use it?
    1. When the plant is infected by fungus and attack by insects (pests)
    2. Before the plant is infected by fungus and attack by insects
    3. Just at the time of infection and attack
    4. NA
    5.
    999. Missing

<u>2: Harvest knowledge and practice information</u>

1. Did you harvest the crop as soon as the crop is mature?
   1. Yes
   2. No
   3. NA
   999. Missing

2. If your answer for Q.1 is yes, how do you know whether the crop is at full maturity or not?
   1. By looking at the color of the seed and plant crop
   2. By measuring the moisture content of the seed crop using traditional techniques
   3. 1 & 2
   4. NA
   999. Missing

3. Do you have especial drying mechanism of premature harvested or rain attached crops?
   1. Yes
   2. No
   3. NA
   999. Missing

4. If your answer for Q.3 is yes, how did you do that?
   1. Immediate open solar drying or air drying at sheds
   2. Solar drying after rain time is over
   3. Keep as it is until trashing
   4. 1 & 2
   5. NA
   999. Missing

5. Did you use threshing to separate the seed of the crop?
   1. Yes
   2. No
   3. NA
   999. Missing

6. If your answer for Q.5 is yes, what types of threshing methods did you use?

1. The legs of animals on the ground
2. Modern machines
3. Using our hands (manually)
4. NA
999. Missing

7. If your answer for Q.6 is the legs of animals, do you think that the crops are contaminated with the fecal matter of animals?

1. Yes
2. No
3. NA
999. Missing

8. After harvesting the crop (cereals and legumes) did you dry to decrease the moisture content to the required level?

a) Yes
b) No
c) NA
999. Missing

9. If your answer for Q.8 is yes, how do dry?

1. Solar drying
2. Professional dryer
3. Drying using fire smoke
4. NA
999. Missing

10. Where is the drying done?

1. On the ground
2. On the road
3. Using plastic sheets on the ground
4. Using especial equipment like drying arena
5. On the roof
6. NA
999. Missing

11. How do you know if the crop (grains and legumes) are really dry or not?

1. Can't be scratched by fingernail
2. Gives a breaking sound when chewed
3. 1 & 2
4. NA
999. Missing

12. Did you sort out any crop after drying before storage?

1. Yes
2. No
3. NA
999. Missing

13. If your answer for Q.12 is yes, what are your sorting criteria?

1. Color
2. Grain size
3. Mechanical damage
4. Mold spoilage
5. Any two criteria
6. Any three criteria
7. All criteria
8. NA
999. Missing

14. What do you do with sorted out grains and legumes?

1. Throw them away
2. Feed to animals
3. Eat themselves
4. Sell
5. NA
999. Missing

15. If your answer to Q. 14 is feed to animals, which animal?

1. Chicken
2. Cow
3. Sheep
4. Goat
5. Pig
6. Horse
7. Donkey
8. NA
999. Missing

3: Household storage knowledge and practice information

1. Where did you store your crops (grains and legumes)?
    1. In "Gota" made from teff straw and mud
    2. In "Gotera" made from wood or bamboo.
    3. In underground pits
    4. In plastic bags overlaying in house
    5. In modern silos
    6. 1 & 4
    7. NA
    999. Missing
2. Did the storage facilities include dry, well-ventilated structures that provide protection from rain, drainage of ground water, protection from entry of rodents and birds, and minimum temperature fluctuations?
    1. Yes
    2. No
    3. NA
    999. Missing
3. Why you didn't use modern storage facilities like silos, etc.?
    1. Costly
    2. Not available
    3. No need
    4. Lack of information
    5. NA
    999. Missing
4. Did you remove old grains or legumes before storage?
    1. Yes
    2. No
    3. NA
    999. Missing
5. Did you treat (disinfect) the storage area before storage?
    1. Yes
    2. No
    3. NA
    999. Missing
6. If your answer for Q.5 is yes, what types of treatment methods did you use?
    1. Smearing with ash
    2. Smearing with cow dung
    3. Neem tree
    4. Smoke of Woira or other
    5. Insecticide fumigation
    6. NA
    999. Missing
7. Did you use any pesticides during storage to prevent insect infestation and rodents attack?
    1. Yes
    2. No
    3. NA
    999. Missing
8. Did you use fumigation during storage?
    1. Yes
    2. No
    3. NA
    999. Missing
9. If your answer for Q.8 is yes, what types of fumigant did you use?
    a. Sulfur dioxide
    b. Carbon dioxide -smoking using plant leaves
    c. Other, specify: ________________
10. Did you use any decontamination methods for mold infected crops?
    1. Yes
    2. No
    3. NA
    999. Missing
11. If your answer for Q.10 is yes, what types of decontamination methods you used?
    1) Roasting
    2) Washing
    3) Sorting
    4) Using chemicals
    5) Mixing with unaffected cereals or legumes

6) Sun drying
7) NA
999. Missing

4: Complementary Food Processing and Handling knowledge and Practice Information at households

1. Where do you collect complementary food ingredients?
   1. Own agriculture
   2. Grain banks
   3. Local markets
   4. Safety net programs
   5. 1 & 3
   6. NA
   999. Missing
2. What qualities do you look for selecting food of your child?
   1. Color
   2. Type
   3. Insect infestations
   4. Moldy
   5. Odor
   6. Any two criteria
   7. Any three criteria
   8. Any four criteria
   9. All
   10. NA
   999. Missing
3. Have you observed mold in any food you used for your child or family?
   1. Yes
   2. No
   3. NA
   999. Missing
4. If your answer is yes for Q.3, what did you do?
   1. Discard
   2. Feed to animals
   3. Eat by roasting
   4. Use for brewing local beverages
   5. Sell
   6. NA
   999. Missing
5. Did you use moldy cereals for brewing local beverages like Tella?
   1. Yes
   2. No
   3. NA
   999. Missing
6. If your answer for Q.5 is yes, do you think that the local beverages made from moldy crops has human health effect?
   1. Yes
   2. No
   3. NA
   999. Missing
7. Did you drink local alcoholic beverages made from moldy cereals at times of lactation?
   1. Yes
   2. No
   3. NA
   999. Missing
8. Did you sort out mechanically damaged cereals and legumes?
   1. Yes
   2. No
   3. NA
   999. Missing
9. If your answer for Q.8 is yes, why do sort out mechanically damaged cereals or legumes?
   1. Susceptible to mold spoilage
   2. Decrease nutritional quality
   3. Decrease organoleptic quality
   4. 1 & 2
   5. 2 & 3
   6. 1 & 3
   7. 1, 2 & 3
   8. NA
   999. Missing
10. What do you think about health effect of feeding moldy food ingredients to animals?

1. Has no effect
2. Has effect
3. NA
999. Missing

11. If your answer for Q.10 has effect, what health problems did you observe so far?
    1. The animal died
    2. Seriously sickened
    3. I don't have any idea
    4. NA
    999. Missing
12. Do you think that feeding moldy food ingredients to animals has an effect on human beings by consuming infected animal products (milk, egg, meat, etc)?
    1. Yes
    2. No
    3. NA
    999. Missing
13. Have you observed any health problem on any person due to eating moldy food?
    1. Yes
    2. No
    3. NA
    999. Missing
14. If your answer for Q.13 is yes, what happened to the infected individuals?
    1. Vomiting
    2. Diarrhea
    3. Headache
    4. Gangrene
    5. Liver disease
    6. Death
    7. NA
    999. Missing
15. What is the most commonly consumed cereal in your area?
    1. Teff
    2. Maize
    3. Wheat
    4. Barley
    5. Oat
    6. Sorghum
    7. Finger millet
    8. Engedo
    9. NA
    999. Missing
16. What is the most commonly consumed legume in your area?
    1. Broad bean
    2. Pea
    3. Chick Peas
    4. Soya bean
    5. Lentils
    6. Haricot bean
    7. Kidney bean
    8. Grass pea
    9. NA
    999. Missing
17. What is the most commonly food you feed your child in your area? (only one answer)
    1. Enjera
    2. Porridge
    3. Kita
    4. Bread
    5. Kollo
    6. Nufro
    7. NA
    999. Missing
18. How do you prepare complementary food for your children?
    1. Soaking
    2. Germination
    3. Fermentation
    4. Drying
    5. Roasting
    6. Milling
    7. Dehulling
    8. All except 1
    9. All except 2
    10. All except 2 & 3
    11. NA
    999. Missing
19. Do you think that some of the processing methods (soaking, germination, fermentation, malting, etc) have contribution on the proliferation of molds?
    1. Yes
    2. No
    3. NA
    999. Missing

20. Did you deshelling or dehulling cereals or legumes for the complementary food processing?
    1. Yes
    2. No
    3. NA
    999. Missing
21. If your answer for Q.20 is yes do you re-wet for ease of deshelling?
    1. Yes
    2. No
    3. NA
    999. Missing
22. Do you think roasting can decontaminate the toxins produced from moldy crops?
    1. Yes
    2. No
    3. NA
    999. Missing
23. Did you dilute moldy ingredients (mix with normal ingredients) while preparing complementary foods?
    1. Yes
    2. No
    3. NA
    999. Missing
24. Do you think that complementary foods can be contaminated from pathogenic microbes if processing do not accomplished in clean area?
    1. Yes
    2. No
    3. NA
    999. Missing
25. If your answer for Q.24 is yes, what problems may happen?
    1. Vomiting
    2. Fever
    3. Headache
    4. Diarrhea
    5. Sudden death
    6. NA
    999. Missing
26. What are the critical times you should wash your hands?
    1. After toilet
    2. Before food preparation
    3. After cleaning children
    4. After taking care of a sick person
    5. All
    6. NA
    999. Missing
27. What did you use to wash your hands?
    1. Clean water only
    2. Clean water and soap
    3. Clean water and ash
    4. Clean water, soap and ash
    5. NA
    999. Missing
28. What would happen if hands are not properly washed after toilet while processing complementary foods?
    1. The food will be contaminated
    2. Nothing happen
    3. Not sure
    4. NA
    999. Missing

29. What did you do if some complementary foods are left over after eating your child?
    a) Discard or give it to dogs
    b) Store it for the next meal
    c) Give it to younger children or grown ups
    d) NA
    999. Missing

5: Complementary Food Ingredients and Products Preparation, Handling and Storage knowledge and Practices at grain banks

1. Where do you collect complementary food ingredients?
   1. Own agriculture
   2. Grain banks
   3. Local markets
   4. Donors
   5. 1 & 3
   6. NA
   999. Missing
2. What qualities are you looking for in selecting complimentary food ingredients?
   1. Color
   2. Type
   3. Insect infections
   4. Odor
   5. Molds
   6. Any two criteria
   7. Any three criteria
   8. Any four criteria
   9. All
   10. NA
   999. Missing
3. What do you do if the ingredients used to make complementary food are not good quality? (Insect infected, moldy, not well dried, premature type, etc)
   1. Discard
   2. Sell it
   3. Use as it is
   4. Dilute (mix) with normal ingredients
   5. Donate to local community
   6. Give as animal feed
   7. Roasted
   8. NA
   999. Missing
4. What is the most commonly used cereal for preparation of complementary food in this grain bank? (Data Collector: Multiple answers possible)
   1. Teff
   2. Maize
   3. Wheat
   4. Oat
   5. Barley
   6. Sorghum
   7. Finger millet
   8. Engedo
   9. NA
   999. Missing
5. What is the most commonly used legume for the preparation of complementary food in this grain bank? (Data Collector: Multiple answers possible)
   1. Broad beans
   2. Peas
   3. Chick Peas
   4. Soya bean
   5. Lentils
   6. Haricot bean
   7. Kidney bean
   8. Grass pea
   9. NA
   999. Missing

6. How do you receive raw ingredients from local mothers at the grain bank?
   1. At separate reception area
   2. At processing area
   3. NA
   999. Missing

7. Did you receive food ingredients cleaned, roasted, dehulled and mixed at household level?
   1. Yes
   2. No
   3. NA
   999. Missing

8. What other ingredients do you use in preparing the complementary foods?
   1. Sugar
   2. Malt

3. Vitamins/mineral premix
4. NA
999. Missing

9. How do you prepare the complementary food products in this grain bank?
1. Cleaning or sorting
2. Soaking
3. Dehulling
4. Drying
5. Roasting
6. Milling
7. Sifting
8. Packing
9. All
10. NA
999. Missing

10. Which milling method did you use in the preparation of complementary foods?
1. Dry milling
2. Wet milling
3. NA
999. Missing

11. Did you clean milling machines between batches of milling?
1. Yes
2. No
3. NA
999. Missing

12. If your answer for Q. 11 is yes, how did you clean the milling machines?
1. By using cloth or sponge
2. Using alcohol
3. Using water
4. NA
999. Missing

13. How often do you wear safety clothes (over coat, hair restraint, shoes, etc) while processing complementary foods?
a) Do not use
b) Some times
c) Always
d) NA
e) 999. Missing

14. Did you store ingredient and prepared complementary foods at the grain bank?
1. Yes
2. No
3. NA
999. Missing

15. How do you keep the food ingredients for longer time?
1. Store at cool, dry and ventilated room
2. By packing in water proof containers
3. By using desiccant like ash to absorb moisture in the storage room
4. NA
999. Missing

16. For how long did you keep (store) them mostly?
1. One week
2. Two weeks
3. Three weeks
4. One month
5. Two months
6. More than two months
7. NA
999. Missing

17. Do you have an established cleaning program at your facility?
1. Yes
2. No
3. NA
999. Missing

18. If your answer for Q.17 is yes, how do you conduct the cleaning?

1. Pre-cleaning or storage places before receiving ingredients
2. Pre-cleaning of milling facilities before processing
3. Cleaning drying area
4. Using insects and rodents proof windows and doors x
5. Has no special cleaning procedure
6. 1 & 2
7. 2 & 3
8. 1, 2 & 3
9. NA
999. Missing

19. How do you store personal items? (Clothes, bags, shoes, etc)?

1. At separate room (lockers)
2. There is no other safety clothes used, and use their own clothes
3. There is no separate room for personal item storage
4. NA
999. Missing

20. How do you control entry of unauthorized personal into your complementary food preparation area?

1. Locked doors
2. Using guards
3. 1 & 2
4. NA
999. Missing

21. What type of information and material do you need that would help you in improving the safety of the complementary food processing and storage?

1. Trainings that help in assuring safety of the raw ingredients and products
2. Equipment's for proper handling and processing of raw ingredients and products
3. Well-equipped storage facility
4. Raw material availing
5. Skilled professionals
6. Vehicles for transporting the raw materials and products
7. Fuel or fire wood for roasting
8. Decorticating or dehulling equipment's
9. Packaging material
10. NA
999. Missing

Annex B: **Conventional method for determining total coliforms and *E. coli*.**

MPN index and 95% confidence limits for various combinations of positive results when various numbers of tubes are used (Inocula of 0.1, 0.01, 0.001g)

3 tubes per dilution			
Combination of positives	**MPN index per g**	**95% confidence interval**	
		Lower	**Upper**
0-0-0	<3	<0.5	<9
0-0-1	3	<0.5	9
0-1-0	3	<0.5	13
0-2-0	---	---	---
1-0-0	4	<0.5	20
1-0-1	7	1	21
1-1-0	7	1	23
1-1-1	11	3	36
1-2-0	11	3	36
2-0-0	9	1	36
2-0-1	14	3	37
2-1-0	15	3	44
2-1-1	20	7	89
2-2-0	21	4	47
2-2-1	28	10	150
2-3-0	---	---	---
3-0-0	23	4	120
3-0-1	39	7	130
3-0-2	64	15	380
3-1-0	43	7	210
3-1-1	75	14	230
3-1-2	120	30	380
3-2-0	93	15	380
3-2-1	150	30	440
3-2-2	210	35	470
3-3-0	240	36	1,300
3-3-1	460	71	2,400
3-3-2	1,100	150	4,800
3-3-3	>1,100	>150	>4,800

ANNEX C: Copy of Ethical Clearance Provided by Ministry of Science and Technology

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የሳይንስና ቴክኖሎጂ ሚኒስቴር
The Federal Democratic Republic of Ethiopia
Ministry of Science and Technology

ቁጥር Ref. No. 3.10/078/2015
ቀን Date Dec 15, 2015

To: Addis Ababa University, Collage of Natural Science, Ethics Committee

Addis Ababa

Re: Evaluating the safety of Complementary Food Produced at Community Level Using Locally Available Ingredients

Dear Sir/Madam//Mr./Mrs./Dr,

The National Research Ethics Review Committee (NRERC) has reviewed the aforementioned project protocol in an expedited manner. We are writing to advise you that NRERC has granted

Full Approval

To the above named project, for a period of **one year (December 15, 2015- December 14, 2016).** All your most recently submitted documents have been approved for use in this study. The study should comply with the standard international and national scientific and ethical guidelines. Any change to the approved protocol or consent material must be reviewed and approved through the amendment process prior to its implementation. In addition, any adverse or unanticipated events should be reported within 24-48 hours to the NRERC. Please ensure that you submit biannual progress report once in six months and annual renewal application 30 days prior to the expiry date.

We, therefore, request you as PI and your esteemed organization to ensure the commencement and conduct of the study accordingly and wish for the successful completion of the project.

With regards,

Yohannes Sitotaw
Secretary of NRERC

CC. Mr. Abebe Ayelign (PI)
_NRERC chairperson

ማነጋገር ቢያስፈልግዎ
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ANNEX D: Food Frequency Questionnaire (FFQ) to assess the possible routes of aflatoxin exposure among young children (1-4 years old) in Ethiopia

C1: Information and Consent

Dear respondent,

We are a member of the Center for Food Science and Nutrition of the Addis Ababa University. We conduct a study to assess the safety of complementary foods prepared from locally available ingredients in your locality. Our goal is to improve the safety of complementary foods prepared from locally available ingredients. You are selected to take part of the study. If you are willing to take part of our study, you will be asked food frequency questionnaire regarding the types of food you give to your child, frequency of consumption per day, per week, and the like. In addition, you will also be asked to give 40 ml of urine from your child as well. Urine recipient will be distributed to each of the participating child a day prior to the day of sample collection. First morning void urine sample will be collected in the next day morning. The urine sample will be stored in an ice box immediately after collection and transported to AAU and stored at deep freezer for a maximum of one month. Then only 15 ml of the urine sample will be transported to Food Analysis Laboratory, Ghent University, Belgium for aflatoxin analysis.

We are, therefore, here to ask for your consent to take part of the study. You are totally free to accept or refuse to Participate in the study. If you accept, we guarantee you that confidentiality of all information collected will be assured. You are free to quit at any moment of the survey, without any prior notice or justification. However, your honest participation will eventually help in assessing the levels of aflatoxin exposure among young children and designing and implementing appropriate interventions to alleviate the problems.

If you have any question, comments, or complaints, you can reach the researchers through the following address.

Abebe Ayelign
Center for Food Science and Nutrition, AAU
Addis Ababa
P.O. Box: 1176
Tel: 0911-105033

Name of the mother for which consent is asked: _______________Signature: _______________

C2: Food Frequency Questionnaire (FFQ)

1. Questionnaire code: ________________Region: ____
2. Kebele: _________________________Woreda: ____
3. Age of the mother/caregivers in years: ____
4. Age of the child in years/months: ____
5. Weight of the child: ____
6. Height of the child: ____
7. MUAC of the child: ____
8. Gender of the child: Male _____________Female _______________________________________
9. Weaning status of the child:
   a) Wholly breastfed
   b) Partially breastfed
   c) Fully weaned

10. Types of food you feed/eat to your child: Thinking about your child's diet over the last one week, please select the responses that best describe how often you feed each type of food and how much of it you feed at a time. Select only one frequency and one serving size per row.

No	How often, did you eat the following to your child for the last one week?	Never	1 time per week	2-3 times/week	1 times per day	2-3 times per day	What was your usual serving size, relative to the following?	1/4	1/2	1 or more
1	**Enjera**: - From teff - From maize - From finger millet - From sorghum -From Teff & maize blend						¼, ½ slice Enjera			
2	**Bread, Kitta**: -From wheat -From maize - From teff -From wheat &maize blend						1/16, 1/8 slice bread. kitta			
3	**Shiro**: -From chickpea -From broad bean - From pea -From grass pea						¼, ½, 1 spoon shiro			
4	**Porridge** from: -Maize, wheat, teff, broad bean, Pea blend -Maize, wheat, teff, broad bean, chickpea blend -Wheat, maize, pea blend -Wheat, maize, BB blend -Wheat, teff, BB blend						1, 2, 3 & more than 3 coffee cup porridge			
6	**Nifro, kollo**: -From maize -From wheat -From sorghum -From barley						1/2, 1, 2 & more than 2 handful Nifro, Kollo			
7	Milk						½, 1, >1 cup of milk			
8	Eggs						½ ,1, 2, >2 eggs			
9	-Meat, -Poultry,						½, 1,>1 slice beef, ½, 1,>1, breast portion chicken			
10	Peanut butter						½, 1, >1 table spoon of peanut butter			

ANNEX E: HACCP based SOPs for preventing the safety of complementary foods produced at community level using locally available ingredients in Ethiopia: pre-harvest to household.

Abstract

Background: HACCP is a systematic approach to food safety management based on recognised principles which aim to identify the hazards that are likely to occur at any stage in the food supply chain and put into place controls that will prevent them from happening. HACCP is very logical and covers all stages of food production from farm-to-table.

Objective: Develop HACCP based SOPs from pre-harvest to household for preventing the CFs from the risks of mold, aflatoxin and microbial contamination.

Methodology: An extensive literature review of the topic was carried out.

Results: All the seven principles of HACCP were described from pre-harvest to houseld practices separately with indicating the critical control points (CCPs) and critical limits (CLs). Instructions (DOS and DONTS) were outlined again from pre-harvest to household practices separately that helps to prevent the CFs from mold, aflatoxin and microbial contamination. Besides, flow diagram that shows the processing steps of CFs at the grain banks were outlined with indicating the CCPs.

Conclusion: This HACCP based SOPs could contribute for the prevention of the risks of mold, aflatoxin, *C. sakazakii*, colifroms, *E. coli* and other pathogens in the locally prepared CFs.

Keywords: HACCP based SOPs; Pre-harvest; Harvest; Storage; Grain banks; Households; aflatoxin; *C. sakazakii*; Coliforms; *E. coli*.

1. Introduction

People have the right to expect the food they eat to be safe and suitable for consumption. Foodborne illness and foodborne injury are at best unpleasant; at worst, they can be fatal. Food spoilage is wasteful, costly and can adversely affect trade and consumer confidence. Effective hygiene control, therefore, is vital to avoid the adverse human health and economic consequences of foodborne illness, foodborne injury, and food spoilage. Everyone, including farmers and growers, manufacturers and processors, food handlers and consumers, has a responsibility to ensure that food is safe and suitable for consumption.

With the introduction of complementary foods (CFs), young children are at risk of exposure to foodborne pathogens and toxins. Contamination of CFs with microbial agents has been recognized as one of the leading causes of diarrhoea diseases and ill health in young children. In addition, contamination of CFs by aflatoxins causes immune suppression, stunting and hepatocellular carcinoma (HCC). Therefore, ensuring the safety of CFs are a goal worth achieving. To ensure the safety of CFs prepared from locally available ingredients in terms of aflatoxin, *C. sakazakii*, coliform and *E. coli*, a hazard analysis critical control point (HACCP) based standard operating procedures (SOPs) are one of the best approaches. The HACCP based SOPs monitor the food chain from farm- to- table, by highlighting the key asepsis controls at each stage.

The farmers, development agents (DA), health extension workers (HEWs), various government agencies and others have shared responsibility of assuring that CFs provided to the young children is of high quality, chemically and microbiologically safe and does not become a vehicle for transmission of a disease outbreak and chronic effects.

The HACCP-based SOPs include the following seven principles:

- ✓ Hazard analysis,
- ✓ Determine critical control points,
- ✓ Establish critical limits,
- ✓ Establish monitoring procedures,
- ✓ Establish corrective actions,

- ✓ Establish verification procedures,
- ✓ Establish record-keeping and documentation procedures.

The HACCP based SOPs will be implemented starting from pre-harvesting the CFs ingredients up to the grain banks where the CFs are processed, and distribute to the children of age between 6-23 months.

2. Describing the CF and its intended use

CF is any food whether manufactured or locally prepared, suitable as a complement to breast milk or to infant formula. CFs can be especially prepared for the infant or can be the same foods available for family members, modified in order to meet the eating skills and needs of the infant. As much as possible CFs are rich in energy and in micronutrients (especially iron, zinc, calcium, vitamin A, vitamin C and folate), free of contamination, without much salt or spices, easy to eat and easily accepted by the infant, in an appropriate amount, easy to prepare from family foods, and at a cost that is acceptable by most families.

WHO recommends the introduction of solid food to infants around six months of age because by that age breast milk alone is no longer adequate to maintain a child's optimal growth. Consuming or giving infants other foods or fluids than breast milk is considered the period of complementary feeding between 6 and 23 months of age. This 18 months' interval is the largest part of the ''1000 days'' encompassing pregnancy and the first 2 years after birth, now viewed as the key window of opportunity for preventing under nutrition and its long-term adverse consequences.

However, during this transition period, introducing the child to CFs, the prevalence of malnutrition increases substantially in many countries and in Ethiopia as well, because of an increase in infections and poor feeding practices. This is maybe the CFs made from locally available ingredients may have the tendency to be contaminated by different contaminants like aflatoxin, *C. sakazakii*, coliforms, *E. coli*, and other microbial pathogens that are the major route of transmission of diarrhea among infants. In Ethiopia, dehydration from diarrhea is a major cause of death in infancy and childhood and 13% of children under age five were reported to have had diarrhea.

Therefore, proper maternal practices regarding the management, preparation, administration and storage of CFs may reduce their contamination. All stages and processes in the food chain must be completed according to correct practices, ensuring that hazards are controlled and eliminated or reduced to acceptable levels.

3. Ingredients and incoming materials used for the production of CFs

Table 1. Ingredients and raw materials used for the production of CFs

Ingredients for CFs		
No.	**Ingredients**	**Example (s)**
1	Cereals	Teff, Maize, Wheat, Barely, Sorghum, Oat, Finger millet
2	Legumes	Pea, Broad bean, Chick pea, Soybean, Haricot bean, Lentils, Kidney bean
3	Additives	Sugar, Fenugreek, Malt, Vitamins/mineral premix
	Incoming materials for CFs	
	Example (s)	
1	Packaging materials	

4. Complementary foods production flow diagram

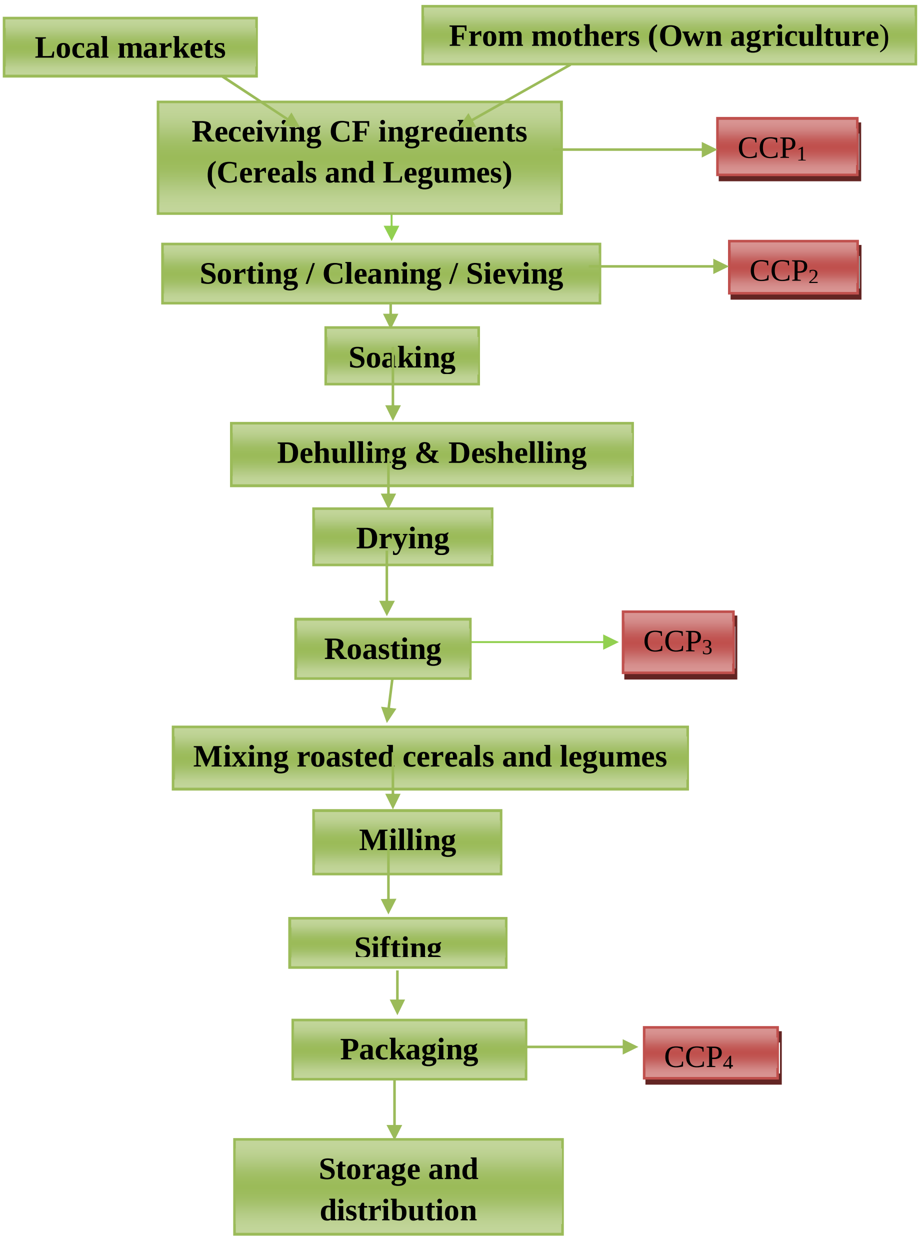

Figure 1. Flow diagram for the processing of CFs at grain banks

5. HACCP based SOPs from pre-harvest to household consumption of CFs

5.1 HACCP based SOPs, pre-harvesting practices

Purpose: To prevent environmental conditions that favour mold infection of cereal and legume crops in the field.

Scope: This procedure applies to farmers (men or women) who engaged in pre-harvest practices of cereal and legume crops.

Keywords: Pre-harvest, Ploughing, Crop rotation, Debris, Mold, Climatic conditions, Seed varieties, Insecticides, Fungicides.

Hazards: Mold is the expected hazard that infects the crop in the field.

Critical control points: Ploughing, removing debris, crop rotation, weed control, preventing insect pests.

Critical limits: Ploughing the land three or more times; at the time of sowing, the farm is free from any debris; planting different crops in the given field every year; every time the crop is free from any types of weed; and no sign of insect pest infestation.

Monitoring procedures: Farmers should follow and observe regularly the critical control points to ensure that they are not out of its critical limits.

Corrective actions: Farmers should take corrective action immediately when monitoring results show some deviations at the CCPs. For example, if some debris is seen at the field during sowing, the debris has to be removed.

Verification and record keeping procedures: Farmers should verify (or check) all the CCPs and all other pre-harvest practices accomplished accordingly. If possible, farmers are expected to document all the pre-harvest practices starting from ploughing the farm land until harvesting.

Instructions (DOS and DON'TS):

- ✓ Plough the farm land properly, three or more times, before sowing the next crop,
- ✓ Undertake a crop rotation schedule to avoid planting the same commodity in a field in two consecutive years,

- ✓ Destroy or remove old seed heads, stalks, and other debris that may potentially serve as substrates for the growth of mycotoxin-producing fungi **(Figure 2)**

Figure 2. Old seed heads and stalks serve as substrates for the growth of aflatoxigenic fungi

- ✓ Apply fertilizer and/or soil conditioners if needed to assure adequate soil pH and plant nutrition to avoid plant stress,
- ✓ Use seed varieties developed for resistance to seed infecting fungi and insect pests,
- ✓ Crop planting should be timed to avoid high temperature and drought stress during the period of seed development and maturation,
- ✓ Avoid overcrowding of plants by maintaining the recommended row and intra-plant spacing for the species **(Figure 3).**

Figure 3. Row planting in a field

- ✓ Control weeds in the crop by use of mechanical methods or by use of registered herbicides or other safe and suitable weed eradication practices,
- ✓ Minimize mechanical damage to plants during cultivation,
- ✓ Minimize insect damage of the crop by proper use of registered insecticides, and other appropriate practices,
- ✓ If you want to use insecticides, use it before the crop is attacked by insects,
- ✓ Pre-infect plants with bio-competitive non-toxigenic fungal strains such as Alfasafe™ to exclude toxigenic fungi.

5.2 HACCP based SOPs, harvesting practices

Purpose: To prevent mold and aflatoxin contamination of cereals and legumes by harvesting at maturity and dried to the required moisture level before storage.

Scope: This procedure applies to farmers (men or women) or others who engaged in harvesting practices of cereals and legumes.

Keywords: Harvesting, Crop maturity, Threshing, Moisture content, Drying, Sorting.

Hazards: Main hazard to be considered is mold known as aflatoxin.

Critical control points: Harvesting, drying.

Critical limits: Harvesting at maturity, the moisture levels of cereals & legumes are less than 12%.

Monitoring procedures: Farmers should follow and observe regularly the critical control points to ensure that they are not beyond its critical limits.

Corrective actions: When there is a deviation from the critical limits at the CCP, i.e. the moisture content of the cereals is not below 12%; keep on drying until the expected moisture level is achieved.

Verification and record keeping procedures: Farmers should verify (or check) all the CCPs and other harvesting practices are accomplished accordingly. If possible, they are also expected to document all the harvesting practices.

Instructions (DOS and DON'TS):

- ✓ Harvest at maturity when the crop has just dried,
- ✓ Avoid mechanical damage to the cereal and legumes and contact with the soil during the harvesting operation,
- ✓ Avoid harvesting cereals and legumes in wet conditions,
- ✓ Freshly harvested cereals and legumes should be cleaned to remove damaged kernels and other foreign matter,
- ✓ Cereals and legumes should be dried soon after harvest, within 24-48 hrs to a moisture content of below 12% before storage,
- ✓ Dry cereals and legumes on black plastic sheets or mat not on bare floor **(Figure 4).**

Figure 4. Avoid drying cereals on a road or on a bare floor

- ✓ Check the moisture level of the cereals and legumes, it is below 12% before storage, using your finger nail or teeth, listening the sounds of the cereal using metal sheet, shake using bottle with salt, and other methods.

5.3 HACCP based SOPs, Storage practices of cereals and legumes at households

Purpose: To prevent mold and aflatoxin contamination of cereals and legumes using appropriate storage facilities.

Scope: This procedure applies to farmers (men or women) or others who engaged in storage practices of cereals and legumes.

Key words: Storage facilities, Mold, Aflatoxin, Sorting, Decontamination methods, Fumigation, Insects, Rodents.

Hazards: Main hazard to be considered is mold known as aflatoxin.

Critical control points: Receiving cereals and legumes.

Critical limits: The moisture level of cereals and legumes should be less than 12% and they are not mechanically damaged.

Monitoring procedures: Men or women who engaged in the storage practices of cereals and legumes should monitor the CCPs, and the storage facility. In addition, they should monitor frequently the status of storage cereals to check insect infestations.

Corrective actions: When monitoring results show a deviation from the critical limits at a CCP, corrective action must be taken immediately. For example, some cereals are infected by mold; you should sort out the moldy cereals and dry the rest further before storage. If cereals are infested by insects discard it or take an appropriate action.

Verification and record keeping procedures: Men or women that are involved in the storage practices of cereals and legumes should verify (check) all the storage practices are accomplished correctly. They also have to document the approximate moisture level of the cereals, the corrective actions taken when deviations occur at the CCPs, in case of insect infestations and the storage structures, in case of rodents' attack.

Instructions (DOS and DON'TS):

- ✓ Make sure that the storage facilities include dry, well-ventilated structures that provide protection from rain, drainage of ground water, entry of rodents and birds,
- ✓ Sort out to discard poor quality and mechanically damaged cereals and legumes before storage,
- ✓ Store cereals and legumes in appropriate structures (improved granaries, silos and jute bags) **(Figure 5).**

Figure 5. Metal silo - an improved maize storage container made of metal sheet

- ✓ Prevent insect pest and rodents attack to prevent spread of mold spores **(Figure 6).**

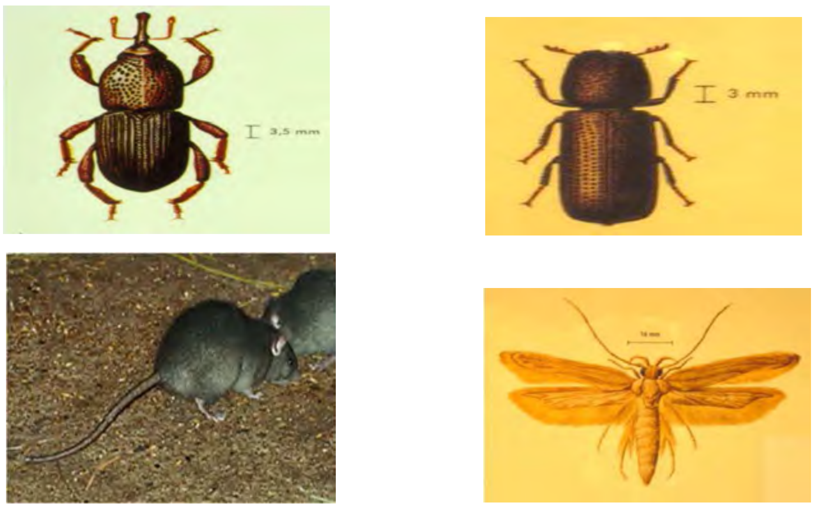

Figure 6. Insects, pests and rodents aggravate mold spread

- ✓ Cereals and legumes to be stored should be dried to below 12% moisture levels and cooled as quickly as possible after harvest,
- ✓ For bagged commodities, ensure that bags are clean, dry and stacked on pallets or incorporate a water impermeable layer between the bags and the floor,
- ✓ Aerate the cereal and legume storage area to maintain proper and uniform temperature levels throughout the storage area. Check moisture content and temperature in the stored cereal at regular intervals during the storage period,
- ✓ Use good housekeeping procedures to minimize the levels of insects and fungi in storage facilities. This may include the use of suitable, registered insecticides and fungicides or appropriate alternative methods.

5.4. HACCP based SOPs; Processing, handling, and storage of CFs at households

Purpose: To prevent contamination of CFs by mold and aflatoxin, *C. sakazakii*, coliforms and *E. coli* using good processing, handling and storage practices.

Scope: This procedure applies to women or caregivers of young children or others who are engaged in processing, handling and storage of CFs at households.

Keywords: CFs**,** CFs processing methods, Cereals, Legumes, Sorting, Decontamination methods, Health impact of aflatoxin, Health impact of pathogenic microbes, Personal hygiene.

Hazards: Main hazards to be considered are mold known as aflatoxin, *C. sakazakii*, coliforms and *E. coli*.

Critical control points: Receiving / selection of CF ingredients, cleaning, roasting and porridge making.

Critical limits: The CF ingredients should be free from mold, insect infestation, mechanically damaged cereals and other foreign substances. Then, roast the CF ingredients at an appropriate time and temperature combinations and make the porridge accordingly.

Monitoring procedures: Mothers or caregivers of the young children should monitor all the procedures used to process CF by giving special attention to the CCPs.

Corrective actions: Mothers or caregivers of young children should take corrective actions if deviation occurs at the CCPs and discard the food produced during the deviation period to ensure the health of the young children.

Verification and record keeping procedures: Mothers or caregivers of young children should verify (or check) and validate the CCPs frequently. If possible, they are expected to document all the processes used to prepare CFs in their homes.

Instructions (DOS and DON'TS):

- ✓ Make sure that the food you used for your child is not moldy,
- ✓ Choose the right color, odour and type of food for your child,
- ✓ Discard if you observe mold in any food you used for your child or family,
- ✓ Don't give moldy cereals and legumes or foods to animals, it has negative health impact to the animal itself and it has human health impact if we consume the products of the given animal (egg, milk or meat) **(Figure 7 and Figure 8).**

Figure 7. Growth reduction in poultry due to aflatoxin in feed at different levels (left normal and right severely affected

Figure 8. Aflatoxin affected chicken liver

- ✓ If you feed contaminated cereals to animals, include mycotoxin-adsorbent clays that tightly binds mycotoxins in the gastrointestinal tract of animals,
- ✓ Don't use moldy cereals for the preparation of local alcoholic beverages, like tella, and others,
- ✓ Sort out moldy, mechanically damaged and insect infested cereals and legumes while preparing CFs,
- ✓ Dehull or deshell cereals and legumes while preparing CFs,
- ✓ Don't mix moldy cereals or legumes to normal ones while preparing CFs,
- ✓ Roasting don't decontaminate aflatoxins produced from moldy cereals or legumes,
- ✓ Roasting does not decontaminate aflatoxins produced from moldy cereals or legumes, thus do not use them for the preparation of CFs
- ✓ Prepare CFs in a clean area to avoid contamination of CFs by pathogenic microbes, like *C. sakazakii*, coliforms, *E. coli* and others,
- ✓ Keep your personal hygiene while preparing CFs to avoid transferring food poisoning microorganisms,
- ✓ Wash your hands properly using clean water and soap or if soap is not available use clean water and ash,
- ✓ Wash your hands after toilet, before preparing food, before and after feeding children, after cleaning children, after taking care of a sick person, after handling raw foods, after handling waste, after carrying out cleaning duties, after handling animals and after any other unhygienic practices.
- ✓ Keep your fingernails short and clean,
- ✓ Be free from any illnesses such as gastroenteritis or flu while preparing CFs,

- ✓ Prepare the required amount of CF for your child at each meal, if some CFs is left over after feeding your child, please give it to elder children or grownups, or give it to animals, or discard it.
- ✓ Don’t keep the left-over CFs for the next meal, because it may be spoiled and cause diseases like diarrhoea.

5.5 HACCP based SOPs; processing, handling and storage of CFs and its ingredients at grain banks

Purpose: To prevent contamination of CFs and its ingredients by mold and aflatoxin, *C. sakazakii*, coliforms and *E. coli* using good processing, handling and storage practices.

Scope: This procedure applies to women who engaged in processing, handling and storage of CFs and its ingredients at grain banks.

Key words: CFs, CF processing methods, Sorting, Cereals, Legumes, Quality of CF ingredients, Milling, Storage facility, Cleaning program, Personal hygiene.

Hazards: Main hazards to be considered are mold known as aflatoxin and microorganisms such as *C. sakazakii*, coliforms and *E. coli*.

Critical control points (CCPs): Receiving CF ingredients (CCP_1), Sorting / Cleaning / Sieving (CCP_2), Roasting (CCP_3), and Packaging (CCP_4).

Critical limits (CLs): The CLs for CCP_1: CF ingredients should be free from mold, insect infested & mechanically damaged kernels, and physical hazards like stone, glass, metals, and other foreign substances. CLs for CCP_2: CF ingredients should be free from mold, insect infested & mechanically damaged kernels, and physical hazards like stone, glass, metals, and other foreign substances. CLs for CCP_3: roasting CF ingredients at appropriate time and temperature combinations. Finally, the CLs for CCP_4: package CF using clean and sanitized material to avoid cross contamination of CFs by *C. sakazakii*, coliforms and *E. coli*.

Monitoring procedures: Monitoring consists of a planned sequence of observations or physical measurements that can be readily recorded at each CCP to ensure that the process is under control. Mothers should monitor or measure the parameters at the CCP to determine whether the critical limits are being respected. If deviation occurs at the CCP, they should take an appropriate action immediately.

Corrective actions: Corrective actions help to correct and eliminate the cause of a deviation, as well as to determine the scope of the problem so that out-of-speciation product can be identified and disposed of. Therefore, mothers working at the grain bank should follow strictly the procedures of CF production so as to eliminate the cause of deviation at the CCPs. In addition, HEW is expected to retrain mothers working at the grain bank if they are not following or implementing the HACCP based SOP correctly.

Verification and record keeping procedures: Verification of the HACCP based SOPs is essential to ensure that all hazards can be controlled and that all controls are operating correctly**.** Mothers working at the grain banks should verify (or check) CLs, monitoring procedures and the corrective action procedures established at each CCP are operating accordingly. They should also have an appropriate documentation and records that are needed to demonstrate the effectiveness of the HACCP based SOPs. Records should be kept for a length of time of at least the shelf life of the product.

Instructions (DOS and DON'TS):

- ✓ Select CF ingredients with an appropriate color, odour and type. Make sure they are non-moldy and free from insect infestations.
- ✓ Don't receive moldy, insect infested and mechanically damaged CF ingredients from mothers. If they brought such ingredients, tell them change and bring another **(Figure 9)**

Figure 9. Receiving moldy, mechanically damaged and insect infested cereals should be avoided

- ✓ Receive CF ingredients from mothers at a separate reception area.
- ✓ Don't receive roasted and dehulled CF ingredients. Ingredients have to be dehulled and roasted at the grain banks to make sure that the ingredients are of good quality.
- ✓ Use other ingredients like sugar and mineral-vitamin premixes (if possible) in preparing CFs.
- ✓ Prepare CFs at the grain bank using the following steps: Cleaning or sorting, dehulling / de-shelling, drying, roasting, milling, sifting, and packaging **(Figure 10)**

Figure 10. Cleaning, sorting significantly reduces aflatoxin contamination

- ✓ Use dry milling methods while preparing CFs at grain banks,
- ✓ Clean milling machines between batches of milling by using cloth or sponge and if possible using alcohol,
- ✓ Keep your personal hygiene clean while preparing CFs at the grain bank,
- ✓ Keep your fingernails short and clean,
- ✓ Be free from any illnesses such as gastroenteritis or flu while preparing CFs,
- ✓ Avoid wearing jewellery while handling and processing CFs,
- ✓ Wear safety clothes like over coat, hair restraint, shoes, etc. while preparing CFs at grain banks. Safety clothes should be clean at the beginning of a work shift

- ✓ Store personal items like clothes, bags, shoes etc in a separate rooms (lockers), if possible,
- ✓ Avoid touching nose, mouth, hair and skin while handling and processing CFs,
- ✓ Store CFs and CF ingredients separately at the grain banks. As much as possible make sure the storage room is cool, dry and ventilated **(Figure 11).**

Figure 11. Better storage structures at the grain banks

- ✓ Store CF ingredients and CFs for a maximum of 2 months at the grain bank,
- ✓ Clean the grain bank facility regularly and establish a cleaning and pest control program **(Figure 12).**
- ✓ Control entry of unauthorized personnel into the CFs preparation area by locking doors and opening windows, and if possible using guards.

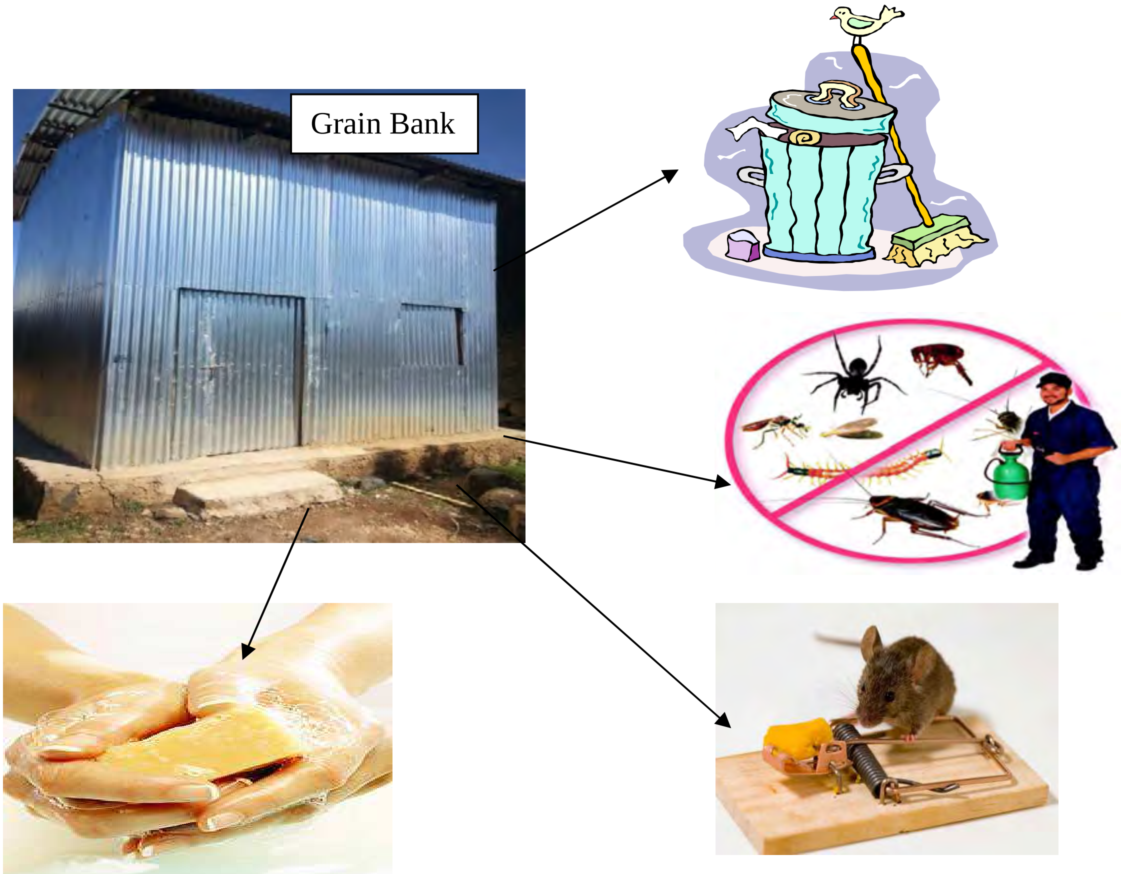

Figure 12. Grain banks should have a cleaning and pest control program

6. Format for record keeping procedures

Format 1. Checklist or record form for receiving CF ingredients

Name of Kebele: ____

Name of mother's giving the ingredients: ____________________________________

Date	Time	Type of cereal or legume	Amount received	Packaging	Hazards identified	Monitoring methods	Corrective actions (if any)	Signature

Format 2. Corrective actions record form

Name Kebele: ____

Name of mothers doing the corrective actions: _________________________________

Date	Time	Critical limit violated	Corrective actions taken	Signature	

Format 3. Cleaning schedule record form

Name of Kebele: ____

Date	Time	Equipment to be cleaned	Cleaning agents to be used	Cleaning method	Cleaning frequency	Signature	Remark

Mothers who has inspected or is involved in this process: _________________

Date: _______________

Format 4. Pest or rodents control monitoring record form

Name of Kebele: ____

Name of mothers involved in this activity: ______________________________

Date	**Location checked**	**Type of baits used to prevent pest or rodents**	**Evidence of infestation or attack**	**Action taken**	**Signature**

Format 5. Personal hygiene inspection form

Name of Kebele: ____

Checked by: ___________________________ Date: _________________

No	**Standard**	Yes	No	Not applicable (NA)	Comments	Date corrected
1	Mothers working at the cereal bank wear proper clothing					
2	Mothers working at the cereal bank wear hair restraints					
3	Finger nails are short, without nail polish and clean					
4	Open sores, cuts, or bandages on hands are completely covered while handling food					
5	Do mothers wear jewellery while handling and processing CF					
6	Mothers take appropriate action when coughing or sneezing					
7	Are mothers free from any illnesses					

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