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CENTER FOR FOOD SCIENCE AND NUTRITION

OCCURRENCE OF AFLATOXIN CONTAMINATION IN MILK AND DAIRY PRODUCTS FROM BISHOFTU AND ITS SURROUNDING

BY

SELAMAWIT TADESSE

ADVISOR:

Dr. ASHAGRIE ZEWDU

Dr. TAREKEGN BERHANU

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By: Selamawit Tadesse

Advisor: Dr.ASHAGRIE ZEWDU

Co-Advisor: Dr.TAREKEGN BERHANU

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Approval by examining board:	Signature	Date
Dr Kaleab Baye (External examiner)		
Dr Abebe Ayelegn (Internal examiner)		01/12/2017
Dr.Ashagrie Zewdu (Advisor)		11/11/2017
Dr Tarekegn Birhanu (Co. Advisor)		11/11/2017
Habtamu Guja (Chairman)		01-12-2017

Declaration

I, the undersigned, declare that this thesis is my original work and that all sources of materials used for the thesis have been duly acknowledged.

Name: Selamawit Tadesse

Signature: _____ Date 11/11/2017

The thesis has been approved for submission by:

Name of Supervisor	**Signature**	**Date**
Dr. Ashagrie Zewdu	_____	11/11/2017
Dr Tarekegn Birhanu	_____	11/11/2017

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Contents

List of abbreviations

Afs	*Aflatoxins*
AFM1	*Aflatoxin M1*
AFB1	*Aflatoxin B1*
AOAC	*Association of Official Analytical chemists*
CV	*Coefficient of variation*
EFSA	*European Food Safety Authority*
ELISA	*Enzyme-linked Immuno sorbent Assay*
ECAE	*Ethiopian conformity assessment Enterprise*
EU	*European Union*
FAO	*Food and Agriculture Organization*
FDA	*Food Drug Administration*
HPLC DAD	*Higher Performance Liquid Chromatography with Diode Array Detector*
HPLC-FLD Detector	*High Performance Liquid Chromatography with Fluorescence*
HPLC-MS	*High performance Liquid Chromatography coupled to Mass Spectrometry*
IARC	*International Agency for Research on Cancer*
KAP	*Knowledge, Attitude and Practice*
LOQ	*Limit of Quantification*
TLC	*Thin-layer Chromatography*
US	*United States*

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Abstract

Aflatoxin contamination of milk in the Greater Addis Ababa milk shed Ethiopia was reported in 2016, indicating a that high probability of contamination of milk coming from the surrounding areas of Addis Ababa. Therefore, the objective of this study was to investigate the occurrence of aflatoxin M1 (AFM1) in milk and dairy products (cheese butter and yogurt) in Bishoftu (Ethiopia). The effect of different dairy production processes to the concentration of AFM1 was also investigated. In addition, KAP (Knowledge attitude and practice) study was conducted on local producer and on employees from dairy industries. Milk (n=108), cheese (82), yogurt (93) and butter (82) samples were taken from both industrial and local producers by purchasing from their shops and the level of AFM1 was determined using ELISA (Enzyme Linked Immuno Sorbate Assay) method. From 366 samples analysed 361 samples were found positive and the maximum value of aflatoxin among all the analyzed samples was found in cheese which is 5.580+0.08μg/kg and the minimum (Not detected) in butter. Under comparing the mean values of each dairy product type of local products with the industrial products all the industrial products have a higher mean value relative to the local one. In addition to this the KAP study shows though the local producers had knowledge on mycotoxin related questions their knowledge specific to AFM1 practice towards reducing the contamination level of AFM1 is not enough. But they have a positive attitude towards reduction of AFM1 level. Unlike the local producers employees in dairy industry had a better practice towards reducing the level of AFM1 contamination. Even though researches have been done about level of AFM1 in milk so far, this study shows that the problem on the occurrence of the toxin still persists. Therefore, the government should establish a program to regularly cheek AFM1 levels and take corrective mitigation measures.

Key words: Aflatoxin M1, Milk, Cheese, Yogurt, ELISA, KAP assessment

Chapter 1

INTRODUCTION

1.1 Background

Aflatoxins are one class of Mycotoxin (Secondary metabolites of microscopic fungi) which are natural contaminant in foods and feeds resulting in human health impact (Leszczyska et al., 2001). It has sub-acute and chronic effects such as liver cancer, chronic hepatitis, jaundice, hepatomegaly and cirrhosis. Since the toxin producing mould was identified as Aspergillus flavus in 1960 and the toxin was given aflatoxin by virtue of its origin (Yitbarek & Tamir, 2014).

Aflatoxins are produced mainly by two species (Aspergillus flavus and Aspergillus Parasiticus) of Aspergillus fungus which specially found in hot and humid climates and Aspergillus flavous which favours the areal part of plant (leaves, flowers) (Filazi and Sireli, 2013).The toxins are naturally occurring and exist at high levels in much of Africas food supply. Some scientists estimate that up to one-third of Africas food supply is infected with aflatoxins at levels higher than the United States (Nowakowski, 2015).

The most frequent aflatoxins are B1, B2, G1 and G2.Aflatoxin B1 (AFB1) is the most toxic and powerful natural carcinogen in human and animal (Hussein and Brasel, 2001; Sengun et al., 2008). Aflatoxin M1 (AFM1) or milk toxin is hydrox-

ilated metabolite of aflatoxin B1 (Creppy, 2002). It is secreted in milk of dairy cattle after consumption of feed contaminated with aflatoxin B1 (Skrbic et al., 2014).International Agency for Research on Cancer has classified AFB1as class one and AFM1 class 2B human carcinogens (Langat et al., 2016).Human exposure to AFM1is due to the consumption of contaminated milk and dairy products (Langat et al., 2016).In Ethiopia, infants are the most susceptibility exposed population due to their high consumption of milk and dairy products in their diet(Zeluta et al., 2009).

Ethiopia has one of the largest livestock population in Africa the national production of milk remains among the lowest in Africa .The total production of milk in Ethiopia is estimated at about 2.7 million tons per annum and the per capital consumption is about 16 kg/year in order to meet the demand, milk production has to grow at least at a rate of 4% per annum (Dup, 2012). Even though food supply issues arent uncommon in Africa, famines caused by drought, flood, or conflict are frequent. This toxin becomes another constant threat to the continent's food security that receives little public attention (Nowakowski, 2015). Ethiopia is already dealing with different food insecurity issues, therefore the trait on this aflatoxin contamination to the countries livestock population which mainly composes of Ethiopians diet will be a critical issue to the countries food security unless it is controlled from the root.

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In order to reduce the public health risk from the consumption of contaminated food and feed regular monitoring of the toxins along the commodity value chain

is mandatory. Developing countries are more vulnerable to such contaminants as there is lack of the capacity to regularly monitor and to regulate mycotoxin levels in foods. This could be due to limited resources and many other alternative priorities. This study has focused on the investigation of the prevalence of aflatoxin in milk and dairy products from Bishoftu and its surrounding. In addition, the knowledge, attitude and practice of the dairy industries and milk farmers in the indicated areas were studied.

1.2 Statement of the problem

A study done on aflatoxin contamination in 2016 of milk in the Greater Addis Ababa milk shed, Ethiopia showed that 29 (26.3%) milk samples of the 100 samples exceeded the 0.5 μg/L limit (Gizachewet al., 2016). This is an indication that there is high probability of contamination of milk coming from the surrounding areas of Addis Ababa. In the study the highest AFM1 content was 4.98 mg/L from DebreZeit and the lowest was 0.028 mg/L from Addis Ababa; which indicates further study should be conducted in this area but there is no report of the development made or indication of the reduction of this contaminant in milk so far. But after the publication of this study the awareness of peoples towards AFM1 has increased and a research on AFM1 level in different commodities has been done. In addition to this acceptable limit value for AFM1 in milk was developed in Ethiopia.

Dairy farms in cities fed commercial feeds which are poorly handled and prone to mycotoxin fungal development which may lead to aflatoxin contamination on both milk and dairy products. Therefore, studying the prevalence of aflatoxin in milk and dairy product samples is fundamental for public health. The knowledge, attitude and practice (KAP) of the farmers and dairy industries plays significant role in reducing the exposure of the public to aflatoxin contaminants. In this regard, KAP study will show the level of awareness of both the farmers in a milk farm and the employees of dairy industries.

In addition to this the levels of AFM1 among different sites could vary due to geographic and climatic differences, but most importantly it could vary due to the differences in feeding systems and farm management practices (Langat, 2016). The conversion of dietary AFB1 into milk AFM1 also varies widely among animal breeds, days, and from one milking session to the next and it is greatly influenced by physiological factors such as diet and health status of animals (Yitbarek & Tamir, 2014) which in turn will show a difference in level of aflatoxin in dairy products which were produced at different areas of a country. Therefore for a country to set its own permissible aflatoxins level it is appropriate to conduct an extensive research considering factors affecting the level of aflatoxin under a desired sample amount.

On the other hand, Milk is a highly variable product that rapidly loses its homogeneity and spoils if untreated. Since milk may be processed in numerous ways, the effects of storage and processing on stability and distribution of AFM1 are of great concern (Yitbarek & Tamir, 2014). Several studies have investigated the distribution/stability of AFM1 from milk to milk products (Iqbal et al., 2013).

Some researchers found out that industrial applications such as production of yogurt, cheese, cream, milk powder and butter do not lead to loss of AFM1, despite AFM1 redistributing differentially into the products resulting from this process (Var and Kabak, 2009),heat processing used in dairy industry i.e. pasteurization and sterilization also has no effect (Prandini et al., 2009). In contrast others observed a variable effect on AFM1 level due to different production processes (Iqbal et al., 2015; Bakirci.2001; Govaris et al., 2002).

As discussed above Variability of reports were observed on the effect of AFM1on cheese butter and yogurt production process and still a general conclusion about the type of production process and its impact on AFM1 level was not made yet.

1.3 Significance of the study

This study focuses on determining the prevalence of aflatoxin in milk from cattle and dairy products (yogurt, cheese and butter). Thereby we can identify which dairy products contain lower or higher level of aflatoxin relative to others.

In general the study is initiated to assess the knowledge and practices of dairy farmers and dairy industries about aflatoxin in milk and dairy products and determine the prevalence and quantify the level of AFM1 in cattle milk and its dairy product from Bishoftu (Ethiopia). This is helpful to identify how the traditional and industrial dairy practices affect the prevalence of aflatoxin in the area.

This study will give us a clear view on the prevalence of aflatoxin in the cattle's milk and its dairy products if it is detected. This will help us on solving food safety problems in milk and dairy production by identifying the sources for the prevalence of aflatoxin.

In addition, limit value for the level of AFM1 for cheese yogurt and butter were not yet set in Ethiopia. Therefore, this study can be used as an input for setting an acceptable limit value for these dairy products in the country.

1.4 Objectives

1.4.1 General objective

To assess the prevalence of aflatoxin contamination in milk and dairy products from and its surrounding.

1.4.2 Specific objective

- To determine the level of aflatoxin M1 in milk and dairy products and find out the prevalence of the toxin in the specified area.

- To assess the knowledge, attitude and practice (KAP) of dairy farmers and employees of the dairy industries regarding aflatoxin contamination and mitigation mechanism.
- To compare the level of aflatoxin M1 milk and dairy products (yogurt, cheese, butter).

Chapter 2

LITERATURE REVIEW

2.1 Aflatoxin

Aflatoxins are a group of mycotoxins that are produced during fungal development as secondary metabolites mainly by members of the fungal genus Aspergilluse specially Aspergillus flavus (A.flavous)and by most if not all strains of Aspergillus parasiticus (A.parasiticus) (Kamkar,2005). These fungal species are naturally occurring contaminants of food and elaborate the toxins under favorable conditions of temperature, relative humidity/moisture and poor storage conditions.

Suitable conditions for development of moulds A. flavus and A. parasitic us in feed are moisture content between 13% and 18%, relative air humidity between 50% and 85% and temperature around 28°C. Factors such as soil humidity and feed damage caused by insects also increase the possibility of development of such moulds (Bilandi et al.,2010). Aflatoxins are more frequently found between latitudes 16° and 35° in warm climate zones and is not common above 45° latitudes (Torres et al.,2014).

Moreover studies revealed that there are 4 major aflatoxins: B1, B2, G1 and G2 plus two additional metabolic products M1 and M2.The two major types of afla-

toxin were named Aflatoxins B and G (blue and green) after the color of their fluorescence under long-wave ultraviolet light .This intense fluorescence forms the basis of most assay techniques for Aflatoxins (Yitbarek and Tamir, 2014) .The aflatoxin M1 and M2 was first isolated from milk of lactating animals hence the M designation. Aflatoxin M1 (AFM1) is 4-hydroxy aflatoxin B1 and aflatoxin M2 is 4-dihydroxy aflatoxin B2 (Buldu et al., 2011).

Aflatoxins are highly toxic, mutagenic, teratogenic and carcinogenic compounds (Bilandi, Varenina and Solomun, 2010) that have been implicated as causative agents in human hepatic and extra hepatic carcinogenesis (Ghanem and Orfi, 2009).It was found that cows metabolize some of the aflatoxin B1(AFB1) in their food which become AFM1, a compound that is closely related to AFB1. The formation of AFM1 occurs in liver and it is secreted into the milk (Unusan, 2006), and may subsequently contaminate other dairy products such as butter, cheese and yogurt.

Of all the mycotoxins, AFB1 is considered to be the most toxic and carcinogenic (Ghanem and Orfi, 2009) and thus has been classified as Group 1 carcinogenic by the International Agency for Research on Cancer (IARC). On the other hand even though the toxicity of AFM1 is lower than that of its parent compound, AFM1 is known forits hepatotoxic and carcinogenic effect (Badea et al.,2004).Since the study is dealing with aflatoxin contamination in milk and dairy products our discussion will focus mainly on AFM1.

2.1.1 Chemical and physical properties

Some important physical and chemical properties of the Aflatoxins are given in Table 2.1 as follows.

Table 2.1: *Physical properties of Aflatoxins*

				UV absorption max (e), nm, methanol	
Aflatoxin	Molecular formula	Molecular weight	Melting Point	265	360 - 362
B1	$C_{17}H_{12}O_6$	312	268 - 269	12,400	21,800
B2	$C_{17}H_{14}O_6$	314	286 - 289	12,100	24,000
G1	$C_{17}H_{12}O_7$	328	244 - 246	9,600	17,700
G2	$C_{17}H_{14}O_7$	330	237 - 240	8,200	17,100
M1	$C_{17}H_{12}O_7$	328	299	14,150	21,250(357)
M2	$C_{17}H_{14}O_7$	330	293	12,100(264)	22,900(357)

The structure of the most frequent aflatoxins are as shown in the Figure 2.1 below B1, B2, G1 and G2.

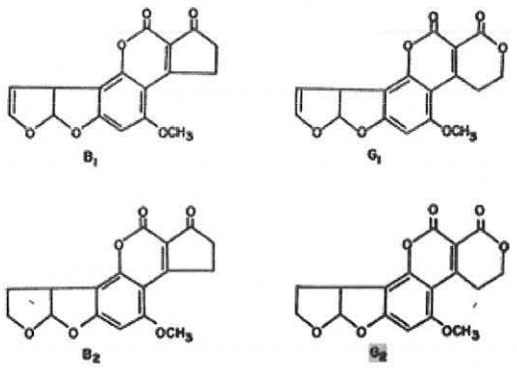

Figure 2.1: Structure of Aflatoxins (Skrbic et al., 2014)

2.1.2 Aflatoxin M1

Aflatoxin M1 (AFM1) is the principal hydroxylated AFB1 metabolite present in milk of cows fed with a diet contaminated with AFB1 and excreted within 12 hours of administration of contaminated feeds (Kang'ethe and Lang'a, 2009). AFM1 in milk has been shown to decline as contaminated feed is withdrawn with no traces of Aflatoxin in milk being detected after 3-4 days of withdrawal (Bilandi, Varenina and Solomun, 2010).

The maximum concentration of AFM1 in milk was found two days after ingestion of AFB1 by dairy cows, but the rate of transfer of aflatoxins is low, 0.3 to 2.2% (Yiannikouris and Jouany, 2002). AFM1 appears to be the major metabolite of AFB1 that has shown appreciable oral toxicity. Its toxicity is considered to be nearly as potent as AFB1 (Skrbic et al, 2014). Carry-over percentage of Aflatoxin from feed to milk was also reported up to 6% have, however, percentage carry-over calculation was best represented by the regression equation as follows (Kan and Meijer, 2007).

Aflatoxin M1 (ng/kg milk) $= 10.95 + 0.787 * (\mu$g aflatoxinB1 intake per day)

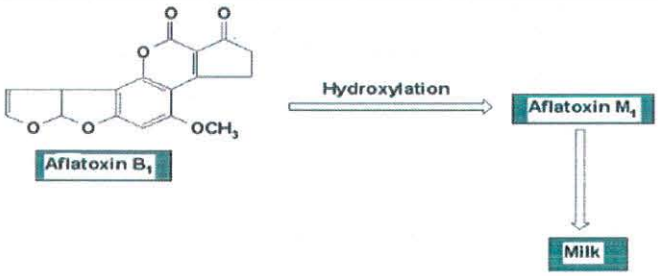

Figure 2.2: Formation of AFM1 as a metabolic products of AFB1 (Fallah,2010)

As shown in the figure 2.2. AFM1 is the main hydroxylated derivative of AFB1 formed in liver by means of P450 cytochrome enzymes and secreted into milk through the mammary gland of dairy cows (Skrbic et al.,2014). Studies by Polan et al. using AFB1, however, have indicated that milk is not the sole excretory route for aflatoxin. It can also be excreted in urine and faces, as well as in the milk of dairy cows (Dhanasekaran et al., 2011; Aycicek et al.,2005).

Distribution of AFM1 in tissues of dairy cows, according to McKinney et al. included 0.1 g/kg in liver, $0.05 - 0.30$ μg /kg in kidney, < 0.05 μg /kg in heart and none in the muscles of cows fed 148.5 mg of AFB1 over a 4-day period (Yitbarek and Tamir, 2014).

2.1.3 Aflatoxin and disease

Aflatoxins have been extensively studied due to their frequent occurrence in foods (especially in developing countries) and their mutagenicity and carcinogenicity (Phillips et al.,2008). It is estimated that about 5 billion people worldwide suffer from uncontrolled exposure to aflatoxin. Aflatoxin-associated health effects pervade Sub-Saharan Africa and East Asia. These effects could be mitigated through effective use of current agricultural knowledge and public health practice (Khlangwiset and Wu,2010).

There are lots of earlier studies reporting the presence of aflatoxins and derivatives in human urine, blood, and human cord blood that apparently can enter the developing fetus in humans and animals (Dhanasekaran et al., 2011). Aflatoxin B1 also excreted in urine and faces, and also in milk of lactating animals either unchanged or as various metabolites (Skrbic et al.,2014).

Aflatoxicosis is the poisoning that can result from aflatoxin (AF) inhalation and ingestion (Bbosa et al.,2013). Two forms of aflatoxicosis have been identified: the first is acute severe intoxication, which results in direct liver damage and subsequent illness or death, and the second is chronic sub symptomatic exposure (Mishra and Das,2010).

2.1.3.1 Acute aflatoxicosis

Acute aflatoxicosis is produced when moderate to high levels of aflatoxins are consumed (Lizrraga-Pauln, 2011) .Outbreaks of acute aflatoxin poisoning is a recurrent public health problem. In 2004, one of the largest, most severe aflatoxicosis outbreaks occurred in Kenya followed by another outbreak in 2005 (Strosnider et al., 2006). The Kenya aflatoxicosis outbreak resulted in 317 acutehepatic failures of which 125 persons eventually died (McCoy et al.,2008). Acute human aflatoxicosis has also been reportedfrom Asian countries such as India (Bhat 1991) and Malaysia (Shephard,2007).

Specific acute episodes of disease include hemorrhage, acute liver damage which manifests as severe hepatotoxicity with a case fatality rate of approximately 25%, edema, problem on absorption and or metabolism of nutrients and alteration in digestion (Strosnider et al., 2006). Young of all species are more susceptible than adults to the effects of aflatoxins . This shows adult humans usually have a high tolerance of aflatoxin, and in the reported acute poisonings, it is usually the children who die (Phillips et al., 2008).

The early symptoms of hepatotoxicity from aflatoxicosis can include anorexia, malaise, and low-grade fever. Acute high-levelexposure can progress to potentially lethal hepatitis with vomiting, abdominal pain, jaundice, fulminant hepatic failure and death (Lizrraga-Pauln, 2011). The symptoms depend on the type of toxin,the amount and duration of the exposure, age, health status and poorly understood synergistic effects involving genetics, dietary status, and interactions with other toxic insults (Dhama et al.,2007).

2.1.3.2 Chronic aflatoxicosis

Chronic exposure to low levels of AFB1 is one of the major risk factors in the aetiology of (HCC) human hepatocellular carcinoma (Shephard et al.,2008); mostly in several regions of Africa and South East Asia, particularly in areas where hepatitis B or C virus infection is endemic (Sun et al.,2010).

It results from ingestion of low to moderate levels of aflatoxins. The effects are usually subclinical and difficult to recognize(Williams et al., 2004). Some of the common symptoms are impaired food conversion and slower rates of growth with or without the production of an overt aflatoxinsyndrome(Lizrraga-Pauln, 2011),which could results in cancers and other irreversible effects (Afsah-Hejri et al., 2013).

2.1.4 Aflatoxin and human health

Aflatoxin exposure of humans is mainly through direct or indirect consumption of contaminated food. Indirect aflatoxin exposure refers to ingestion of milk and dairy products with AFM1 carried over from contaminated feed (Afsah-Hejri et al., 2013). Consumption of biotransformation products of animal like eggs and tissues from animals fed with mycotoxin-contaminated feed is another source of indirect exposure (Dhama et al.,2007).

The direct respiratory exposure to AFB1-contaminated dust results in tumors in the respiratory tract. After inhalation of AFB1-contaminated dust, lung cells and nasal mucosal epithelial cells biodegrade AFB1 and subsequently B1-DNA adducts are formed (Afsah-Hejri et al., 2013). The reported outbreaks of aflatoxicosis in men were due to the consumption of staple foods such as maize and not due to the consumption of groundnut. It was reported that groundnut meal contaminated with aflatoxin caused Indian childhood cirrohsis and liver cancer (Mishra and Das,2003).

In human chronic toxicity of AFs results in cancers and other irreversible effects (Bbosaet al.,2013). The symptoms for acute toxicity are jaundice, diarrhea, depression, low-grade fever, anorexia, liver damage, and decreased essential serum proteins synthesized by the liver(Ongoma,2013). In severe cases, it leads to death. The acute lethal dose for adult humans is 10 to 20 mg and the estimated acutelethal dose for children is approximately 3 mg (Afsah-Hejri et al.,2013). Clinical symptoms of acute aflatoxicosis in human are: vomiting, high fever, highly colored urine, tremors, convulsion, cerebraledema, coma, elevated serum transaminases, hypoglycemia, and fatty degeneration in the liver and kidneys (Agag 2004).

Aflatoxins have also been reported to interfere with nutrition in a dose response relationship between exposure to aflatoxin and rate of growth in infants and children (Bbosa et al.,2013). Therefore, in humans, the aflatoxin exposure and the toxic effects of AFs on immunity and nutrition may combine to negatively af-

fect health factors (including HIV infection) that account for more than 40% of the burden of disease in developing countries where a short lifespan is prevalent (Moturi,2008).

Hepatocellular carcinoma (HCC, or liver cancer) is the primary disease associated with aflatoxin intake. This disease is the third-leading cause of cancer death globally with extremely poor prognosis. (Bbosa et al.,2013) Estimates suggest that more than 600 000 people die of liver cancer worldwide each year with a majority of cases occurring in China, south-east Asia and sub-Saharan Africa (Wild & Gong, 2010); where the major risk factors of chronic infection with hepatitis B and C viruses(HBV and HCV) as well as dietary exposure to aflatoxins are a problem (Wogan et al., 2012). According to Wild and Gng 2009, aflatoxin appears to be a more potent carcinogen among HBV chronic carriers than among non-carriers.

2.1.5 Aflatoxin and animals

Effects of aflatoxin consumption are similar in all animals but susceptibility varies with breed, species, age, dose, length of exposure and nutritional status (Richard, 2007). Aflatoxins generally affect growing poultry, piglets, pregnant sows and calves. Adult cattle, sheep, and goats are relatively resistant to the acute form of the disease but are susceptible if toxic diets are fed over long period (Dhama et al., 2007).

After entering the body, the aflatoxins are absorbed across the cell membranes where they reach the blood circulation. They are distributed in blood to different tissues and to the liver, the main organ of metabolism of xenobiotics (Bbosa et al.,2013). Other water-soluble conjugated metabolites, AFB1 degradation products and non-conjugated metabolites are excreted into the blood circulatory system and distributed systemically. Eventually, these residues are referred to milk, eggs, muscle and edible tissues (Martins, 2007). AFM1 is one of those metabolic derivatives that taint milk. The animal organism usually produces those metabolic products as an auto detoxification system (Skrbic et al.,2014).AFB1

mainly affects birds, pigs and other monogastric animals.Ruminants are less vulnerable to aflatoxin ingestion. Experimental animal evidence suggests that chronic exposure to aflatoxins may lead to impaired immunity and reduced uptake of nutrients from the diet too (Strosnider, 2006).

Clinical signs of aflatoxicosis in animals include gastrointestinal dysfunction, reduced reproductively, reduced feed utilization and efficiency, anemia and jaundice. Nursing animals may be affected as a result of the conversion of aflatoxin B1 to the metabolite aflatoxin M1 excreted in milk of dairy cattle (Talebi, 2011). Symptoms of prolonged to moderate exposure to aflatoxins may be reflected in a decline in feed consumption and production (growth and production of eggs and milk (Iheshiulor et al., 2011). It can also affect the quality of milk and milk products, and represent a risk for the presence of AFM1 as derived from AFB1 consumed by lactating females (Hussein & Brasel, 2001).

Aflatoxin causes a variety of symptoms depending on the animal species. However, in all animals, aflatoxin can cause liver damage, decreased reproductive performance, reduced milk or age production, embryonic death, teratogenicity (birth defect),tumour and suppressed immune system function, even when low levels are consumed (Akande et al.,2006). For most animal species, the range of LD50 values is 0.5 to10 mg/kg body weight (Afsah-Hejri,2013).

In chronic aflatoxin poisoning, most of the effects are still referable to hepatic injury, but on a milder scale. Hepatic pathology includes a yellow to brassy color, enlarged gall bladder, dilute bile, histological signs of fatty changes in the hepatocytes and bile duct proliferation(Whitlow,2005). Frequently the signs of chronic aflatoxins are so protean that the condition goes undiagnosed for long periods. Chronic aflatoxin poisoning however, is the manner in which animals are most frequently affected and the economic consequences are often considerable (Lizrraga-Pauln, 2011). Inchronic case liver is the main target. Aflatoxins act as a DNA intercalating agent by binding to guanine bases and leading to cell death or its transformation into a tumour (Yiannikouris and Jouany,2002).

The risk posed by aflatoxin depends on the level and type of aflatoxin indiet, the strain of animal, and its nutritional status(Mishra and Das, 2003).In poultry, beside inappetance, weight loss, decreased egg production, leg and bone problems, poor pigmentation, fatty liver, kidney dysfunction, bruising and death, suppression to natural immunity and susceptibility to parasitic, bacterial and viral infections can occur (Bbosa et al.,2013).Acute aflatoxin poisoning provokesmajor signs of liver lesions, leading to congestion and bleeding. Aflatoxicosis causes fatty acid accumulation in the liver, kidneys and heart and may be responsible for encephalopathies and oedemas. The animal may die within a few hours or days (Yiannikouris and Jouany,2002).

2.1.5.1 Cattle

In cattle, AFB1 reduces milk production and impairs the growth of calves. In pregnant animals, abortions are common. In calves, there is retarded growth rate and they may succumb within 48 hrs of acute exposure (Dhama et al., 2007).

The signs of acute toxicosis in dairy and beef cattle, include anorexia, depression, dramatic drop in milk production, weight loss (Akande et al.,2006), lethargy, gastrointestinal dysfunctions such as abdominal pain, bloody diarrhoea, decreased feed intake and efficiency; jaundice, abortion, hepatoencephalopathy, blindness, walking in circles, ear twitching, frothy mouth, photo sensitization, bleeding and death (Bbosa et al.,2013)The level of aflatoxin exceeds 100 ppb is considered toxic for cattle and signs of chronic exposure are dry nose, harsh coat, in appetence, abdominal pain and diarrhoea (Dhama et al., 2007).

2.2 Animal feed management as a cause for aflatoxin contamination

The contamination of feed stuffs with mycotoxins is of increasing concern as changes in agricultural practice and probably climatic changes seem to have increased the prevalence of mycotoxin contamination (Gremmels, 2008).Contami-

nation of feeds with mycotoxins accounts for significant economic losses in animal husbandry, as well as in undesirable trade barriers for raw materials and consumable products (Wayne and Bryden, 2014).

In developing countries the primary concern with mycotoxin contamination of the food supply chain is human health and the impact on animal health and production is the second major concern (Wayne and Bryden, 2014).Whereas in developed economies, where mycotoxin contamination in the food and feed chains is tightly regulated to reduce human and animal exposure, the additional costs to the producer and/or the consumer to meet the economic burden of regulating the food and feed supply is the major mycotoxin concern. This is followed by the impact on animal health and production(Shephard,2008). Gizachew et al. 2016 report that, the major contributor to the aflatoxin contamination in the periurban dairy value chain of Addis Ababa was noug (Guizotiaabyssinicaor Niger seed) cake (widely used as cattle feed); which results contamination AFM1in milk .

Removal or inactivation of the toxins often proved to be quite difficult or not economical. mycotoxin control in the field is however quite hard to be executed science weather conditions play a pivotal role in fungal growth and mycotoxin formation, and cannot easily be controlled (Kan and Meijer,2007). Therefore recommended systems such as GMP (good manufacturing practices) for feed production or storage places and GAP (Good Agricultural Practices) at the farm should be implemented and monitored to minimize or avoid the growth of AF in feed.

2.3 Overview on dairy products

2.3.1 Composition and Nutritional values of dairy products

Milk and dairy products are recognized as important sources of nutrients in human diets, providing energy, high quality protein, essential minerals, vitamins and antioxidants (Lock and Bauman, 2004). Some of the minerals found in cows milk are Ca (calcium), P (Phosphorus) and Zn (Zinc) are mainly in casein bound

(about 75, 60 and 98%, respectively) while Mg (Magnesium) and K (Potassium) are mainly in soluble form (about 70 and 90%, respectively) (Lucas et al.,2006). Due to its high nutritional value milk is considered to be a perfect natural food for consumers of all age groups. Additionally, certain milk lipids such as butyric acid are known to present anti carcinogenic properties (Tsakiris et al., 2013).

Now a day the number, the potency, and the importance of bio active compounds in milk and especially in fermented milk products are probably increasing. They include bio active peptides, oligosaccharides, and organic (including fatty) acids (Bergamoet al.,2003). Some of them are normal milk components, others emerge during digestive or fermentation processes (Lock and Bauman, 2004). The composition of milk has a dynamic nature, and it varies with stage of lactation, age, breed, nutrition, energy balance and health status of the (Maijala, 2000). Fermented dairy products and probiotic bacteria decrease the absorption of cholesterol. Whey proteins, medium-chain fatty acids and in particular calcium and other minerals may contribute to the beneficial effect of dairy foods (Microbiol,2008).

Cheeses vary in their composition in terms of their nutrients such as proteins, lipids, carbohydrates, minerals, calcium, phosphorus and vitamins A and B. Cheese is one of the most nutritious food products known to contain 48% of fat and 23-25% protein. Calcium, which is present in large amounts in cheese, has beneficial effects in fighting hypertension, osteoporosis and dental problems (Cruz et al., 2011).In addition to calcium, other elements, such as bioactive peptides, attribute to cheeses potential for anti-carcinogenic properties(Lucas et al.,2006). Due to their nutritional properties, cheeses play an important role in the nutrition of people of all ages and they are one of the best food products, not only for their nutritive value, but also for its large variety of shapes, tastes and as consumers healthy foods of lower fat and calorie content (Sandrou and Arvanitoyannis,2000).

The nutrient composition of yogurt is based on the nutrient composition of the milk from which it is derived (Sa nchez-Segarra et al.,2000; Mckinley,2005), which

is affected by many factors, such as genetic and individual mammalian differences, feed, stage of lactation, age, and environmental factors such as the season of the year (Adolfsson et al., 2004).Other variables that play a role during processing of milk, including temperature, duration of heat exposure, exposure to light, and storage conditions, also affect the nutritional value of the final product (Ahmadi et al., 2011). In addition, the changes in milk constituents that occur during lactic acid fermentation influence the nutritional and physiologic value of the finished yogurt product (Adolfsson et al., 2004).

The final nutritional composition of yogurt is also affected by the species and strains of bacteria used in the fermentation, the source and type of milk solids that maybe added before fermentation, and the temperature and duration of the fermentation process (Sandrou and Arvanitoyannis,2000). The ability of non-pathogenic intestinal microflora, such as LAB (Lactic acid bacteria), to associate with and bind to the intestinal brush border tissue is thought to be an important attribute that prevents harmful pathogens from accessing the gastrointestinal mucosa (Lourens-Hattingh andViljoen,2001).

2.3.2 Manufacturing process of dairy products

2.3.2.1 Milk pasteurization

Pasteurization is a process named after Louis Pasteur (1822-1895).This process has developed to fight back the spread of bovine tuberculosis and other diseases through milk. During pasteurization the milk is heated to a temperature to kill pathogenic bacteria. This also kills many non-pathogenic organisms and thereby extends its storage ability of the milk (ILRU, 2016).

Different time temperature combinations are recommended but the most usual is 72°C for 15 seconds followed by cooling to below 10°C . This is normally referred to as High temperature short time treatment (HTST). HTST is carried out as a contentious process using a plate heat-exchanger to heat the milk and hold section to

ensure that the milk is completely pasteurized. But during butch pasteurization where milk quantity is too small to justify the use of plate heat-exchanger fixed quantity of milk are heated at 63°C for 30 munities. The milk is then cooled at 50°C using cold water before package (ILRI, 2016).

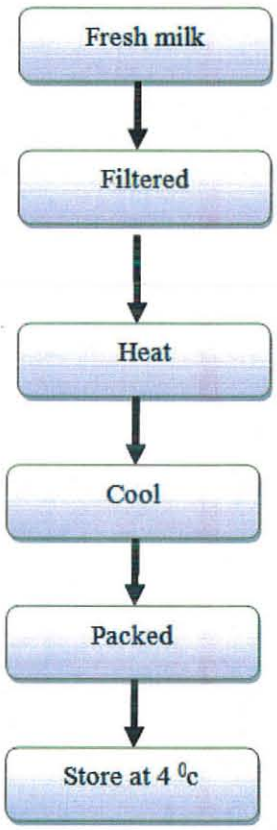

Figure 2.3: Milk pasteurization process

As shown in the Figure 2.3 above after fresh milk is collected from milk producers it is filtered using cloth filtration then heated for 30 minutes at 63°C. After heating its temperature will be lower by cooling it using a container filled with water. Immediately after cooling the pasteurized milk is packed in an appropriate container and kept in a refrigerator at 4°C (ILRI, 2016).

2.3.2.2 Yogurt making process

Row milk under normal condition develops acidity and its acidity reaches to a PH of 3.8 to 4.2. Bacteria in milk are responsible for this acidic PH and the acidity helps to preserve the other milk constituents. Yogurt products are made of milk under controlled fermentation.

This is achieved by establishing the desired micro-organism in the milk and by maintaining the milk at a temperature favorable to the fermentative organism (ILRU, 2016).

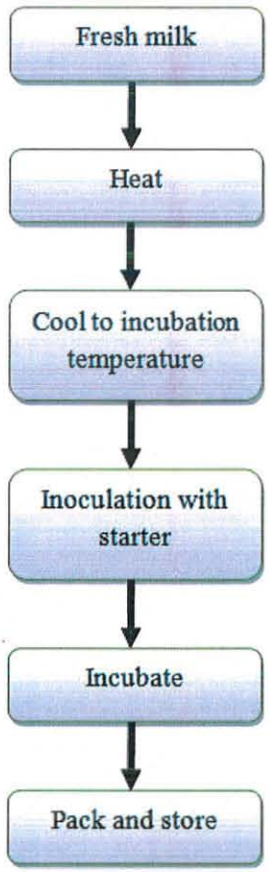

Figure 2.4: Yogurt manufacturing process

As shown in the figure 2.4 the first step in yogurt production process is heating (85°C) which helps to kill pathogens and spoilage organisms and to provide a

cleaner medium in which the desired micro-organisms can be established. Heating also removes air from the milk, resulting a more favourable environment for the fermentative organisms, and denatures the whey proteins, which increases the viscosity of the product (ILRI, 2016).

After heating the milk should be cool to the desire temperature for the microbial culture before starter (microbial culture) is added otherwise the high temperature will kill the starter itself. In addition, care must be taken during inoculation of the culture to avoid contamination. The bacteria used are thermopile, the most useful being lactobacillus bulgaricus and streptococcus thermosphilous with an incubation temperature of 42°C and 4-6 hour incubation time. After incubation time is completed the product will be packed with an appropriate container and kept in to a refrigerator (ILRI, 2016).

2.3.3 Cheese making process (Ethiopian Traditional Cottage Cheese)

Cheese preparation is based on milk fermentation; when the milk is sour enough (PH 4.5-4.6) which could take up to three days the dry matter is separated from the liquid. There are two main separation processes: one is cloth filtration the other uses centrifugal force. The first is ancient traditional method; the second has been developed for large quantity production (ILRI, 2016).

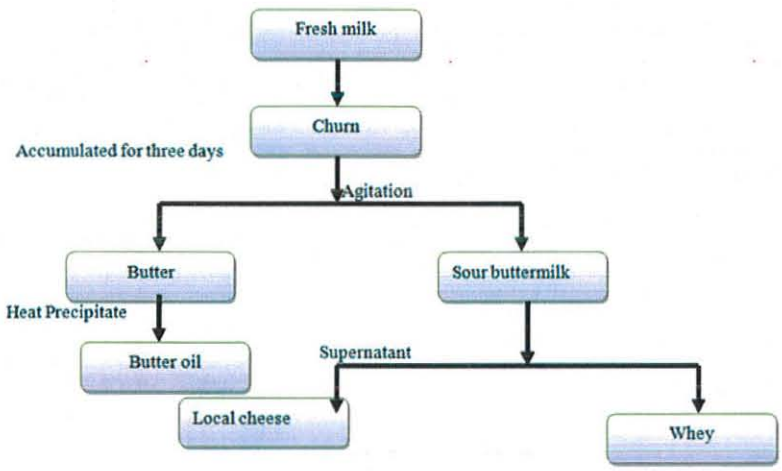

Figure 2.5: Local cheese making process

The Figure 2.5 above shows the cheese making process starts from sour butter milk which is a by-product of butter making process. This milk is heated to 85°C to destroy most of the bacteria present and also to increase yield through precipitation of the way protein. A solution of lemon juice is used to precipitate the curd and this lemon juice is added with contentious stirring and the steering contentious up to 3 minutes after addition of the juice (ILRI, 2016).

Finally after settling it for 15 minutes the curd separates from the way by draining it through sieve or couth stirring the curd while draining prevents excess matting. Next to separation process the curd transferred to a container lined to cheese clause and covered by folding it over the cheese cloth. For pressing a wooden follower fit neatly inside the mold and after pressing overnight by placing metal weights on top of the wooden follower the final cheese product can be pot in to a refrigerator as it is or cut in to a suitable size and packed (ILRI, 2016).

2.3.4 Butter making process

Butter making process also started from sour milk. Under normal condition the milk become sour after keeping it for four to five hours. This souring process has an advantage on retarding the growth of undesirable microorganisms and by making the milk easier to churn (ILRI, 2016).

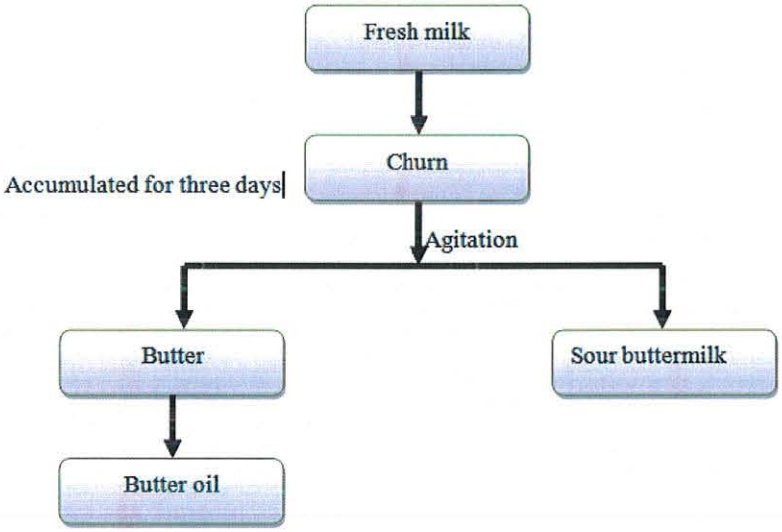

Figure 2.6: Butter making process

As shown in the Figure 2.6 above milk collected from raw milk producers will be collected in a churn for several days by adding fresh milk to the milk already accumulated. The churn may hold up to 20 litters (half of its volumetric capacity) and the amount of milk churned ranges 4-10 litters. Butter is then made by agitating the milk until butter grains form. The churn is then rotated slowly until the fat coalesces in to a continuous mass. Sour milk is normally churned between 15 to 26 °C depending on the environmental temperature. At lower temperature churning time decreases but the optimal churning temperature is between 15 and 17 °C (ILRI, 2016).

When the desire grain size is obtained the butter milk is drained off and the butter washed several times in the churn. Each washing is done by adding only as match water as is needed to float the butter and then turning the churn a few times. The water is the drained of as a general rule two washings are enough but in very hot weather three may be necessary before the water comes away clear. In hot season the coldest water available should be used for washing, and in a cold season about 2 to 3 °C colder than churning temperature should be used (ILRI, 2016).

After washing is completed salt is added to the butter (16gm salt/Kg) most commonly using dry-salting method in which dry salt is sprinkled evenly over the butter. The butter is then either rolled out 8 to 10 times or ridged with the spatulas to remove excess moisture. Finally the butter is packed in a clean container and stored in a cold place (ILRI, 2016).

2.4 Occurrences of aflatoxin in dairy products

Dairy products are food products from mammals milk if the mammal consumes aflatoxin contaminated feed (AFB1) its milk will be contaminated by AFM1 there by the dairy product from this milk is expected to have the toxin (Prandini et al., 2009). This results from thegenerally assumption that neither storage nor processing determine reduction of AFM1 content (Mohammadi ,2011). But the source of contamination in the milk cant only be from the feed it could also be from direct contamination with the fungi aflatoxin during handling and processing (YitbarekandTamir 2013).

Different studies were conducted on the occurrence of AFM1 in milk and dairy products. (Gizachew et al 2016) reported that, from a total of 100 raw milk samples collected from milk producers in Addis Ababa and its surrounding areas (27 from Addis Ababa, 23 from Debre Zeit, nine from Sebeta, 31 from Sendafa and 10 from Sululta) all the milk samples were contaminated with AFM1 with a median value of 0.092 μg/L .The highest AFM1 level was 4.98 μg/L from Debre Zeit, and the lowest was 0.028 μg/L from Addis Ababa. According to Unusan (2006) a total of 129 samples of commercial UHT whole milk collected from Turkey 75 (58.1%) milk samples were contaminated with the mean value of 0.108 μg/L.

On the other hand in Brazil, (Iha et al. 2011), analyzed a total of 123 samples of dairy products (cheese, yoghurt, and dairy drinks) collected during 2010 and AFM1 was detected ($>$ 3 ng/kg) in 49 cheese samples (84%). Thirty nine of the cheese samples (67%) were contaminated with AFM1 in the range from 10 to 304 ng/kg. AFM1 was detected ($>$ 3 ng/kg) in 62 yoghurt and dairy drink samples

(95%), and 47 of the yoghurt samples (72%)and 10 of the dairy drinks (83%) were contaminated with AFM1 at levels ranging from 10 to 529 ng/kg and 10 to 50 ng/kg, respectively. Lopez et al. (2003) observed no micotoxin above the tolerated levels of AFM1 in liquid milk and powdered milk (0.05 μg/L, in Argentina) on 77 various types of milk samples.

In general there are many reports of AFM1 contamination in milk anddairy products in different countries including reports from Turkey (Bakirci,2001; Var and Kabak ,2009; elik, Sarmehmetolu, and Kpll , 2005), Iran (Kamkar,2005; Ghazani,2009; Alborzi et al.,2009; Heshmati and Milani, 2010; Tajkarimi et al.,2008; Sadia et al., 2012;Sohrabi .N and Gharahkoli .H ,2016; Fallah,2010), Brazil (Shundo et al.,2009;), India (Rastogi et al.,2004;Thirumala-Devi et al.,2002), North Africa (Libya) (Elgerbi et al.,2004).

From the above mentioned reports on the level of AFM1 from different countries we can see that the occurrence of the toxin in dairy products is a serious issue. Especially in developing countries. And from this research the occurrence of toxin in Ethiopia, at the specified area of the country (Bishoftu) was investigated.

2.5 Stability of Aflatoxin M1 in Milk and Milk Products

Several studies have in vestigated the distribution/stability of AFM1 from milk to milk products (Iqbal et al.,2015). Some researchers found out that Govaris et al. 2002; Prandini et al., 2009). Industrial applications such as production of yogurt, cheese, cream, milk powder and butter do not lead to loss of AFM1,despite AFM1 redistributing differentially into the products results from this process (Var and Kabak,2009),heat processes used in dairy industry i.e. pasteurization and sterilization also has no effect on AFM1 level (Prandini et al., 2009).

According to Prandini et al.,2009 greatest losses occurred in concentration and spray drying of milk; concentrations remained about the same by heat treatments,

storage at low temperature, during yogurt and buttermilk production; but the concentrations increased on cheese making process and only small amounts went into butter. Evidence has been obtained that aflatoxin M1 binds to the milk protein casein (Aksoy et al., 2016).Another evidence on the increase of AF could be the growth of aflatoxigenic molds on these products. Contamination of this type can occur in any dairy product that is improperly processed or stored (Yitbarek andTamir , 2013).

Some studies also reported that the toxin was not degraded during conversion of artificially or naturally contaminated milk into pressed curd during cheese-making process but, the distribution of AFM1 between curd and whey can be variable (Oruc et al., 2006;). Similarly several authors reported on the influence of yogurt manufacture on AFM1 content. Iha et al. (2013) observed that there is no effect on level of AFM1during fermentation and the effects on AFM1 of cheese and yoghurt storage are minimal; in contrast others observed a significantly decreased AFM1 content (Iqbal et al.,2015) and a 13% higher level (difference of AFM1 level was not statistically significant) of AFM1 in yogurt (Bakirci. 2001) related to milk. According to Govaris et al. (2002),this decrease in AFM1 levels may be attributed to factors such as low pH, the formation of organic acids or other fermentation byproducts, and even the presence of Lactobacillus.

Even though different researches have been conducted in this area the result on the concentration level of aflatoxin in the dairy products vary from one research over the other and gives a contradictory idea, therefore as mentioned above it is difficult to generalize that milk processing to dairy product neither reduce nor increase AFM1 content.

In this study different samples of milk and dairy products will be collected from the market, where their source of production is known, in Debrezeit area and their level of aflatoxin will be analysed. Therefore; it will give some clear idea on the effect of different milk processing techniques on the level of AFM1 on the dairy product. In addition to that it will give a hint on the process of establishing

an action level of AFM1 in milk and dairy products in the future in Ethiopia.

2.5.1 Stability of AFM1 in animal milk

Milk is a highly variable product that rapidly loses its homogeneity and spoils if untreated. Since milk may be processed in numerous ways, the effects of storage and processing on stability and distribution of AFM1 are of great concern (Kaniou-Grigoriadou et al., 2005) once it was found that aflatoxin could be present in raw milk, researchers studied effects of processing on contaminated milk and Variable effects have been reported concerning different heat treatments.

Bakirci (2001) observed that pasteurization caused a decrease in the level of AFM1 at the rate of 7.62%, however, decrease was not significant. Similarly Deveci (2007) observed a significant decrease on the level of AFM1 (12.4% and 9.1% respectively) after pasteurization of two milk samples having 4.9μg and 3.5 μg of AFM1at 72 °C for 2min.IncontrastGovaris et al.(2002) reported the content of AFM1 in milk is not reduced by pasteurization, sterilization or freezing milk or dairy products.

Different researches ware also made on the effects of storage and processing on stability and distribution of AFM1.They observed that detectable AFM1 decreased by 11 to 25% after 3 days at 5 °C, 40% after 4 days at 0 °C, and 80% after 6 days at 0 °C. Whereas, freezing at -18 °C for 30 days resulted in an apparent loss of 14%, with 85% lost after 53 days and suggested less degradation of AFM1 at -18 °C with insignificant loss after 53 days (YitbarekandTamir 2013). Incontrast Prandini et al.,2009 observed no effect on storage at low temperature.

2.5.2 Stability of AFM1 in cheese

Cheese is the most potent source of aflatoxin among dairy products because AFM1 is being associated with the casein fraction in milk is somewhat concen-

trated in cheese (Tavakoli et al.,2012). Studies showed that the concentration of AFM1 is about 3 fold higher in many soft cheeses and about 5 fold higher in hard cheeses than the milk (Ardic et al.,2009). Occurrence of AF in cheese can be due to three possible cause; AFM1 may be found in the milk of animals that are fed with aflatoxin B1 (AFB1) containing feed, synthesis of AF (B1, B2, G1 and G2) by A. flavus and Aspergillus parasiticus growing on cheese and occurrence of these toxins in dried milk used to enrich the milk used to make cheese (Elkak et al.,2012; Oruc et al., 2006).

Variable results were also observed on the impact of cheese production process on the level of AFM1 (Sengun et al., 2008) similar to the milk as mentioned above and it was pointed that it is due to extraction technique, methodology, type and degree of milk contamination, differences in milk quality, expression of the results, the presence of a small portion of curd in whey, which could influence AFM1 concentration, and the cheese manufacture process (Kamkar,2006;Fallah et al., 2009).

In addition to thisthe type of analytical method with its analytical error and possible differences between naturally and artificially contaminated milk (Yitbarek andTamir , 2013)and seasonal changes; milk produced during hot seasons was less contaminated with AFM1 than the milk produced during cold ones (Fallah et al., 2011)may explain conflicting data reported by various researchers.

According to Oruc et al. (2006), during cheese production process, due to the high affinity of AFM1 to the casein and its semi polar characteristics, the higher percentage of AFM1 present in raw milk had passed to curd (52% & 58%) than whey (47% & 40%).Similarly some researchers also reported that the greatest proportion of AFM1 was in the curd ranging between 66-80% (sengun et al., 2008; Kaniou-Grigoradou et al., 2005; Kamkaretal.,2008).

In contrast according to several studies, 86 %, 61%, 66% and 60% of AFM1 present in raw milk had passed to whey during cheese production (Deveci ,2007); which is similar to a report from Lpez et al.(2001)where 60% of the AFM1 in whey and

40% in cheese was found from milk artificially contaminated with AFM1.These conflicting ideas could also arise due to the difference in manufacture and/or analytical techniques, contamination type and level of toxin, cheese type and chemical composition of cheese (Sarmehmetoglu et al., 2004).

From the above reports it can be seen that during cheese production process the AFM1 primarily present in the milk does not totally move to the curd or whey rather it distributes between them at a different level of concentration. Therefore; avoiding contamination appears to be the only practical way to ensure the safety of milk and milk products for human consumption (Deveci ,2007).

2.5.3 Stability and occurrence of AFM1 in Butter

During butter processing AFM1 mainly soluble in the aqueous phase of milk or adsorbed to casein particles (Prandini et al.,2009); information of several studies show that a small ratio of AFM1 in milk is carried-over to cream, and yet a smaller proportion to butter. The remainder of AFM1 in milk, however, remains in skim milk and buttermilk (Atasever et al.,2010).

Therefore, cream contains less AFM1 than milk and butter contains less AFM1 than cream (Bakirci,2001) As a result of the associated effects of these factors, less AFM1 occurs in the lipid phase (butter and cream) because it is concentrated in the serum phase and protein fraction. However, it is probable that good manufacturing practices and good storage prevented butter samples from getting moldy (Aksoy et al., 2016).

2.5.4 Stability of AFM1 in yogurt

During yogurt fermentation an increased AFM1content was reported by Bakirci (2001), which possibly results from a more complete recovery of AFM1from yogurt than milk. On the other hand Govaris et al. (2002) reported a significant decrease of AFM1 levels in all yoghurt samples from those initially present in

milk. This decrease in AFM1 levels might be attributed to factors such as low pH, formation of organic acids or other fermentation by-products, or even to the presence of lactic acid bacteria. The low pH during fermentation alters the structure of milk proteins such as the caseins leading to formation of yoghurt coagulum. The change in casein structure during yoghurt production may affect the association of AFM1 with this protein (Mohammadi, 2011).

As to AFM1 stability over storage of yogurt Govaris et al. (2002) also observed that during refrigerated storage, AFM1 was rather more stable in the yoghurts with pH 4.6 than with pH 4.0. The percentage loss of the initial amount of AFM1 in milk was estimated at about 13 and 22% by the end of the fermentation, and 16 and 34% by the end of storage for yoghurts with pHs 4.6 and 4.0, respectively. And it was explained that the reduction of AFM1 in yoghurt during storage period might be due to the oxidation of glucose by glucose oxidase as catalyst. In contrast others observed no reduction of AFM1 in yogurt stored for 7 days at 7°C (Yitbarek andTamir , 2013) .

These differences in results might be explained by the differences in extraction procedures, concentration of toxin, time elapsed before analysis, storage temperature, milk contaminating method, variability in composition of milk, or differences in the behaviour of starter cultures used in preparing the yoghurt(Elsanhoty et al., 2014).

2.6 Permissible worldwide aflatoxin M1 levels

To protect consumers from these health risks, many countries have adopted regulations to limit exposure to mycotoxins (Dohlman,2003). But due to the influence of economic considerations the regulatory limits throughout the world may vary from one country to another (Aiad and El-Makarem,2013).

In developed countries, human populations are protected because regular surveillance keeps contaminated foods out of the food supply. Unfortunately, in countries where populations are facing starvation, or where regulations are either

non-existent or unenforced, routine ingestion of aflatoxin is very common (Van Egmond, 2007).

On a worldwide basis, at least 99 countries had mycotoxin regulations for food and/or feed in late 2003. In Africa 15 countries are with known where most regulations exist for aflatoxins; Morocco had the most detailed mycotoxin regulations. But in Europe 39 countries are with known regulations and the regulations exist for aflatoxins, ochratoxin A and patulin , in addition regulations are in development for several Fusarium toxins in foods, baby foods and feedstuffs(van Egmond and Jonker,2005).

The following table shows the permissible level of AFM1 sated by different countries

Table 2.2: *International legislation on AFM1 in milk and dairy products for human consumption (Kaniou-Grigoriadou et al., 2005)*

Country	Raw milk (μg/kg)	Dairy products (μg/kg)
European Union	0.05	0.05
Austria	0.05, 0.01 (pasteurized infant milk)	0.02 (butter), 0.25(cheese),0.4(powdered milk)
France	0.05, 0.03 (for children < 3 years)	-
Switzerland	0.05	0.025 (milk whey and products), 0.25(cheese), 0.02(butter)
Bulgary	0.05	0.10 (powdered milk)
Rumania	0	0
Check Republic	0.05	-
USA	-	0.05
Brazil	-	0.50(fluid milk),5.0 (powdered milk)
Argentina	0.05	0.50(milk products)
Honduras	0.05	0.25 (cheese)
Nigeria	1	-
Egypt	0	0
Turkeyc	0.05	0.25 (cheese)

In addition in Morocco;most detailed mycotoxin regulations from African countries;the maximum level of AFM1 for Milk (product), Milk powder, for infant under 3 years and cereals are 0.05, 0.5, 0.03, and 30 μg/kg (Zinedine and Maes,2009).As

shown in Table 2.2,given the public health concerns, the EU continues to maintain the maximum level of 0.05 μg/kg in milk AFM1 and 0.025 μg/kgin dairy foods for infants (Berg, 2003). The similar to European Community, Codex Alimentarius Commission prescribed that the maximum level of aflatoxin M1 in milk and milk products should not exceed 0.05g/kg(Rokhi,2013). On the other hand in Egypt, the Ministry of Health established that fluid milk and dairy products should be free from AFM1 (Aiad and El-Makarem, 2013) ,which is similar with Rumanias regulation (Kaniou-Grigoriadou et.al., 2005).

Similarly Africa, Asia and Latin America, apply a maximum level of 0.05μg AFM1/kg in milk and 0.5μg AFM1/kg in milk is applied in the United States, several Asian, European countries and in Latin America(Berg, 2003). Ethiopia sated 0.5 ppb (0.5 μg/kg) as maximum level for AFM1 in milk (ECAE,2016). In Iran maximum level for AFM1 in yoghurt, butter and buttermilk, cheese and Kashk, dried milk and milk are 0.050, 0.020, 0.250,0.5 and 0.05 μg/kg respectively (Fallah et al., 2011).

Nevertheless, it is important to unify regulations of permissible aflatoxin levels in order to avoid risks and health problems derived from importing and exporting contaminated food and food security and aflatoxin contamination.In this research the concentrations of AFM1 in raw milk and some dairy products in Bishoftu, Ethiopia was determined and these levels of AFM1 was compared with maximum AFM1 limits adopted by Egyptian, Eu (Europian Union) Codex, Ethiopia and US(United states of America)regulations.

2.7 Treatment and prevention of diseases caused by aflatoxins

Most aflatoxicos is results from eating contaminated foods (Yitbarek andTamir,2013). Unfortunately, except for supportive therapy (e.g. diet and hydration) there are almost no treatments for aflatoxin exposure. However, there have been described few and specific methods for veterinary management of mycotoxicosis (Lizrraga-Pauln, et al., 2011).

In a primary prevention trial, with the goal to reduce aflatoxins in the diet. A range of interventions includes planting pest-resistant varieties of staple crops, attempting to lower mold growth in harvested crops, improving storage methods following harvest, and using trapping agents that block the uptake of unavoidably ingested aflatoxins (Kabak et al.,2007). In secondary prevention trials, one goal is to modulate the metabolism of ingested aflatoxin to enhance detoxification processes, thereby reducing internal dose and subsequent risk (Lizrraga-Pauln, et al., 2011).

As mentioned above the best way would be the prevention of mycotoxin formation in the field of its first place, which is supported by proper crop rotation and fungicide administration at the right time (Zaki et al.,2012). Pimaricin (natamycin) is commonly used as anantifungal agent in some European countries and recently the Food and Drug Administration has permitted its use on certain cheeses in the United States; also impregnating the wrapping or packaging material with fungicides or fungistatic chemicals (e.g sorbate for cheeses under Federal Standard of Identity 0.2-0.3% is allowed) has helped control mould growth on cheese surfaces (Sengun,2008)

Most efforts to address the mycotoxin problem involve analytic detection, government regulation, and diversion of mycotoxin-contaminated commodities from the food supply (Lizrraga-Pauln, et al., 2011). Basic research on the biosynthesis and molecular biology of aflatoxins has been a priority because a full understanding of the fundamental biological processes may yield new control strategies for the abolition of aflatoxin contamination of food crops.

In addition, by giving awareness that mycotoxin production is dependent on a number of factors including water activity, temperature, substrate, strain of mould, gas composition, the presence of chemical preservatives, and microbial interactions (Sengun et al.,2008); farmers should follow a good agricultural practice, including an appropriate drying of crops after harvest, avoidance of moisture during storage and moisture, insect and rodent control during storage. On

applying the above practices, it is possible to prevent damages to the crop and animal feed, which would promote aflatoxin development.

2.8 Aflatoxin contamination and food safety

At both peri-urban sites, feed resources for dairy cattle were observed to be acquired in two wayson-farm production (For improved forage species and crop residues) and through purchases, in case of oil seed cakes, hulls, mill house scraps and native grass hays (Geleti et al.,2014). The most common ingredients in feeds were wheat bran, noug (Guizotiaabyssinicaor Niger seed) cake, pea hulls and maize grain. Dairy farmers also widely used agro-industrial by-products including Brewers dry yeast from beer factories (Gizachew et al., 2016).

Wheat bran, maize grain and Brewers dry yeast had relatively low levels of aflatoxin contamination, but noug (Guizotiaabyssinicaor Niger seed) cake is a common feed ingredient in the greater Addis Ababa milk shed. Which is highly sensitive to storage environment relative to others. Therefore, Nougcakes were highly contaminated with AFB1 and the presence of noug cake in the feed significantly increased AFB1 in feed and AFM1 in milk (Gizachew et al., 2016).

Over 90% of dairy farmers did not know that milk could be contaminated with aflatoxins through feeding of aflatoxin contaminated feed (Gizachew et al., 2016).In addition to this milking is done by hand which makes it highly susceptible to contamination. The type of feed used, the unsafe food preparation practice, the low awareness in aflatoxin toxicity and some research results on the level of aflatoxin in milk and feed is an indication for the presence of high level of aflatoxin contamination in the peri-urban dairy value chain of Addis Ababa which requires urgent response to reduce human and animal exposure to these toxins.

There is a need to increase awareness of aflatoxins including good manufacturing practices (GMP) which focused particularly on food safety and milk hygiene and support risk mitigation practices along the dairy value chain. Policymakers need to support the dissemination of information about simple risk-reduction

measures including proper drying, sorting, sanitation, proper storage and insect management, among others. In particular the humidity, the packaging type and the storage area of noug (Guizotiaabyssinicaor Niger seed) cake should regularly be controlled/monitored by the owners and/or the responsible food safety regulatory body in Ethiopia. Above all there is a need for doing further research on the dietary manufacturing practices in Ethiopia which leads to aflatoxin contamination which in turn puts in question the food safety issues in milk and other dietary products.

Since one of the objectives of this study is to examine the possible cause of aflatoxin through KAP study through interviewing the appropriate peoples and looking their practices onsite, the outcome of this study will give a clear idea on their level of awareness on aflatoxin contamination which can be used as a guideline on selecting a type of training for them when an awareness creation program launches in Debrezite in the feature.

2.9 Aflatoxin contamination and food security in Ethiopia

Milk production and consumption by the rural population in Ethiopia is limited. According to CSA (2010), of the total annual milk production at national level in 2009/10, 84.7 percent was used for household consumption, seven percent sold, 0.3 percent used to pay wages in kind and the remaining eight percent was used for other purposes such as the production of butter and Ayib (Ethiopian cottage cheese) (Yilma et al.,2011).Poultry is raised by most households in both rural and urban settings but the poultry products are mostly consumed by urban households (Bereda,et al.,2014).

Food insecurity is widespread and severe in Ethiopia, affecting as much as 45% of the population. It is the result of a combination of environmental and man-made factors. Food insecurity is chronic, but the population is at risk of acute food insecurity in the event of drought and other climatic shocks (FAO, 2008). More than seven million Ethiopians are chronically food insecure and receive regular sup-

port from the cash- and food-for-work Productive Safety Net Programme (PSNP), while in any given year several million others face shocks requiring emergency assistance (Lavers,2011).

Even though food supply issues arent uncommon in Africa, famines caused by drought, flood, or conflict are frequent. But now a days there is another constant threat to the continents food security that receives little public attention: Food-borne toxins known as aflatoxins (Nowakowski, 2015) which is an urgent health problems related to agriculture include fungal toxins (mycotoxins) in crops and animal source foods (such as milk and egg) (Grace et al., 2015).

In addition to exposure from crops, metabolites are transferred to breast milk and animal products, especially dairy. Thus, aflatoxins pose health risks to humans when consumed through crops, animal- source food and during breast feeding. The impacts of aflatoxins on animal health have consequences on food production and livelihoods of farmers (Lindahl et al., 2014). The toxins are naturally occurring and exist at high levels in much of Africas food supply. Some scientists estimate that up to one-third of Africas food supply is infected with aflatoxins at levels higher than the United States (Nowakowski, 2015).

Ethiopia is already dealing with different food insecurity issues, therefore the trait on this aflatoxin contamination to the countries livestock population which mainly composes of Ethiopians diet will be a critical issue to the countries food security unless it is controlled from the root.

Therefore, in conducting an analysis on the level of aflatoxin in milk and dairy products in Debrezite area where most of the population of all age consume daily, this research can investigate the trait level of aflatoxin in that specific area and by finding the possible cause of the prevalence it will help to minimize the food insecurity issue in that area.

2.10 Aflatoxin contamination and its Economic Impact

Aflatoxins impose burdens on human health, animal health and productivity, the agriculture sector and the wider economy (Wayne and Bryden, 2014). Annual economic costs of mycotoxins to the U.S. agricultural economy were estimated to average $1.4 billion and the annual cost of regulatory enforcement, testing and other quality control measures was $466 million USD annually, a study estimated that African food exporters lost $670 million per year by not meeting EU safety standards alone (Grace et al., 2015).

However, Xiong and Beghin showed that the standards set by the EU had no significant trade impact on Africa exports. Their findings concluded that the trade potential of African exporters is more constrained by domestic supply issues such as quality, consistency and delivered cost than by limited market access. African exports were already declining and African exporters would likely not have met the earlier less restrictive standards either (Grace et al., 2015).

This shows that aflatoxins cause major economic disruptions through their impacts on trade and livestock production, therefore a better understanding of health impacts of aflatoxins, the risk posed from different products in different contexts and the economic costs of aflatoxins across human health, the livestock industry, the agricultural industry and would trade motivate towards greater investment in aflatoxin assessment and control should be a major concern for African countries.

Therefore, this research can be a starting point for Ethiopian FDA to set a limit values on the level of aflatoxin in milk and dairy products so that those product can be sold with no restrictions of health issues both in the local market and across world trade.

2.11 Different methods used for detection of aflatoxin

Many analytical and immunological methods are available for estimation of AFM1 in milk (Tavakoli et al.,2012; Krska et al., 2008). Commonly used analytical methods for the determination of aflatoxins include thin-layer chromatography(TLC), high-performance liquid chromatography (HPLC), gaschromatography (GC), LCMS (liquid chromatography-mass spectroscopy),LCMS/MS and (ELISA) immunochemical methods such as enzyme linked immune sorbent assay (Heshmati and Milani,2010; Kamkar, 2006;Krska et al., 2008). GC methods are used less frequently because, being replaced by LC-MS/MS (Pereira et al., 2014).

Since toxicity occurs at very low concentrations the sample must be extracted with different extraction methods and cleaned-up prior to detection techniques, if reliable results are to be obtained (Pereira et al., 2014). Most widely used extraction procedures include SPE (Solid phase extraction) and IAC (Immunoaffinity) clean-up methods will become of increasing importance as sample preparation techniques prior to instrumental analysis(Cavaliere,2006). Immunoaffinity cleanup techniques with high-resolution chromatography showed the most selectivity for aflatoxin analysis. Recently, advances using tandem or mixed selectivity immunoaffinity cartridges have demonstrated the feasibility of multi target mycotoxin assays (Krska et al.,2005)

After the extraction of the analyte (aflatoxin) from the sample and applying a clean-up procedure to remove interferences, the next step is the detection of aflatoxins (Pereira et al., 2014); three main types of assays have been developed. These include biological, analytical and immunological methods. The biological methods were used when analytical and immunological methods were not available for routine analysis. Biological assays are non-specific and time consuming and are qualitative in nature (Zheng, 2006).

Among the available detectors, the most frequently used are (PDA) Photo diode array, (UV) UV visible, and (Fl) Floresence which have a particular application in

the field of mycotoxins (Krska et al., 2008). LCMS has all the HPLC advantages for trace level detection and confirmation, especially for complex matrices and it can obtain qualitative data concerning the identity of aflatoxin (Hussain, 2011). The great potential of LCMS/MS for screening large amounts of samples for the presence of a number of mycotoxins has recently been demonstrated. Immunoassays that deliver quantitative or semi quantitative results, still represent the most frequently used rapid methods (Stefanovic et al.,2015)

2.11.1 Thin-layer chromatography (TLC)

Thin-layer chromatography consists of a stationary phase immobilized on a glass or plastic plate and a solvent acting as a mobile phase. The sample, either liquid or dissolved in a volatile solvent, is applied in the form of a spot on the stationary phase. Then the chromatographic plate is placed vertically in a solvent reservoir and the solvent moves upthe plate by capillary action. (Hussain, 2011). TLC provides a cheaper alternative to LC-based methods and has an important role, especially in developing countries, for surveillance purposes and control of regulatory limits (Krska et al., 2008).

When the solvent front reaches a certain limit of the stationary phase, the plate is removed from the solvent reservoir. The separated spots are then visualized with ultraviolet light or by spraying with a suitable reagent. The contents of a sample can be identified by running standards simultaneously with the unknown spots. The different components in a mixture move up the plate at different rates due to differences in their partitioning behaviour between the mobile liquid phase and the stationary phase. TLC can identify and quantify aflatoxins at levels as low as 1μg/ g (Hussain, 2011).

Screening methods are mostly based on thin layer chromatography, which is a very effective and simple technique (Krska et al.,2005). TLC was the most widely used chromatographic technique applied to mycotoxins because of its relatively simple, fast, and inexpensive properties; however, it has some disadvantages,

such as low sensitivity, high detection limit, and lack of potential for automation. Consequently, it is now almost replaced by the HPLC techniques (Stefanovic et al.,2015)

2.11.2 High performance thin-layer chromatography (HP-TLC)

There is lack of precision associated with TLC procedures due to the introduction of possible errors during the sample application, plate development, and plate interpretation steps (Krska et al.,2005). High performance thin-layer chromatography methods improve the precision by automating the sample application and plate interpretation steps. This technique is less commonly used as compared to HPLC, which is more sophisticated as compared to this. (Hussain, 2011)

2.11.3 High performance liquid chromatography (HPLC)

In HPLC, a liquid mobile phase or solvent is used to move the sample through the column. An immobilized liquid stationary phase is packed in the column. The analytic is then partitioned between the two phases as it passes through the column and thus leading to the separation of compounds due to the difference in partitioning coefficients. Two types of HPLC methods are commonly used i.e., normal phase chromatography and reversed phase chromatography. In normal phase chromatography, a polar stationary phase e.g. silica gel and a non-polar solvent e.g. hexane is used (Pereira et al., 2014).

Whereas reversed-phase chromatography (RP-HPLC) employs non-polar stationary phase e.g., C-8 or C-18 hydrocarbons and polar mobile phase e.g. water, methanol or acetonitrile. In HPLC, detection is mainly accomplished by using ultra violet (UV) detector, diode array detector (DAD) or a fluorescence detector (FLD) (Krska et al., 2008; Pereira et al., 2014). Fluorescence detection utilizes the emission of light (435 nm) from molecules that have been excited to higher energy levels by absorption of electromagnetic radiation (365 nm) for aflatoxins (Hussain, 2011).

Fluorescence detection has superior sensitivity than other detection systems and sometimes derivatization of the analyte has to be performed which enhances the sensitivity (Beltrn et al.,2011) Fluorescence detection is possible in the range of microgram/kg. Choice of detector usually depends on the nature of the sample. HPLC system gives result in the form of chromatogram (Hussain, 2011).

2.11.4 Liquid chromatography with mass spectrometric detection (LC-MS)

It is one of the most advanced techniques, time-consuming and it requires expert knowledge. LCMS can provide decisive advantages in performing identification as well as determination of analytes at trace levels(Hussain, 2011).The great advantage of LC-MS methods is the possibility of simultaneous identification and quantification of almost all the mycotoxins at low levels, without derivatization, which is mandatory in GC methods(Pereira et al., 2014). However, the coupling of both techniques is really efficacious if a suitable combination of sample preparation, chromatographic conditions and interface is selected (Cavaliere et al.,2006).

In LC-MS, the HPLC effluent enters an ionization chamber via a nebulizer. There are several techniques for ionization, namely electrospray, thermo-spray, chemical and fast atom bombardment. Fragmentation takes place in a collision chamber. The fragments then enter the high vacuum region of the mass spectrometer, where detection takes place. Several set-ups are available for optimal identification and quantification (Hussain, 2011).

Ion trap instruments are more suitable for identification than triple quadruple instruments with higher MS power (Stefanovic et al., 2015), whereas triple quadruple instruments provide better information for quantification with faster scanning and higher sensitivity(Beltrn et al.,2011).In Selection-Ion-Monitoring (SIM) mode, detection can be made at pico-grams levels (Hussain, 2011). The great potential of LC-MS/MS for screening large amountsof samples for the presence of a

number of mycotoxins and their degradation products has recently been demonstrated (Krska et al.,2005).

2.11.5 Enzyme-linked immunosorbent assay (ELISA)

It is the most commonly used immunochemical methods relative to the other types (immunuaffinity column assay), due to its simplicity sensitivity and adaptability;it is a quick, reliable and cost effective for estimation of AFM1 (Fallah et al,2009) and has been included in the official collection of test procedures by the German Federal Board of Health (Unusan,2006).

There are two types of enzyme-linked immune sorbent assay, which are direct competitive enzyme-linked immune sorbent assay and indirect competitive enzyme-linked immunosorbent assay. In direct competitive enzyme-linked immunosorbent assay method, specific antibody is coated to a solid phase such as a microtiter plate, whereas in indirect competitive enzyme-linked immunosorbent assay method, toxin-protein conjugate is coated onto the microtiter plate.

In aflatoxin analysis, direct competitive enzyme-linked immunosorbent assay is used. The enzyme-linked immunosorbent assay is detection and quantification of an antigen (aflatoxin) in a sample busing an enzyme labeled toxin and antibodies specific to aflatoxin. The enzyme-linked immunosorbent assay is based on antigen-antibody reaction. Antigen is that substance which can elicit production of antibodies when introduced into warm blooded animals. Whereas antibodies are glycoproteins which are produced as a result of an immune response, after introduction of antigens, leading to the production of a specific antigen-antibody complex (Hussain, 2011).

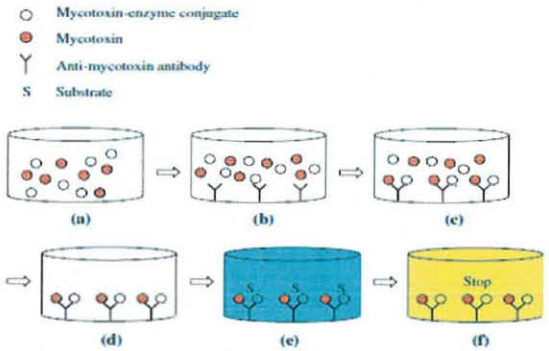

Figure 2.7: Principle of competitive ELISA for mycotoxin analysis.

(a) Sample mixed with conjugate; (b) mixed content added to antibody coated well; (c) mycotoxin binds to antibody in the 1st incubation; (d) unbound materials are rinsed away in the washing step;(e) Substrate is added to develop colour; (f) stop solution is added to stop the reaction

As shown in the figure 2.7 above, the direct competitive enzyme-linked immunosorbent assay, specific antibodies for aflatoxin are coated on to the wells in the microtiter strip. The test samples or aflatoxin standards are added to the wells. After incubation and washing, enzyme conjugate (a conjugate of aflatoxin and bovine serum albumin is attached with an enzyme molecule, such as, horseradish peroxidase or penicillinase or alkaline phosphatase) is added to the wells. Free aflatoxin and aflatoxin enzyme conjugate compete for the aflatoxin antibody sites in the wells. Washing step removes any unbound enzyme conjugate. Then substrate/chromogen is added to the wells and incubated (Hussain, 2011).

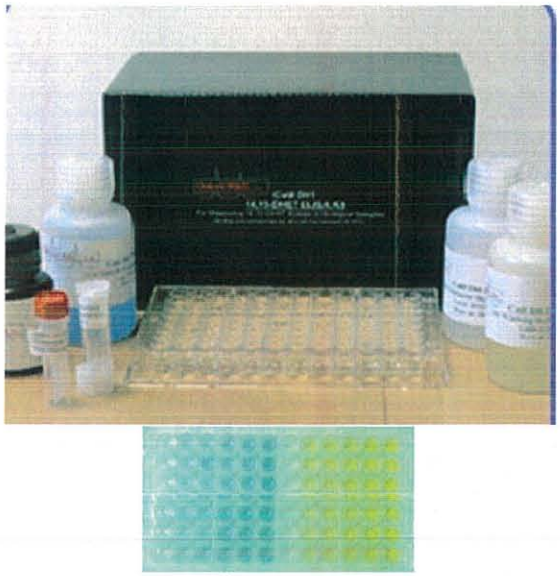

Figure 2.8: Elisa Kit containing all the reagents and standards

As it can be seen on the plate in the Figure 2.8 above at the right, the bounded enzyme conjugate converts the colourless chromogen into a blue product. The stop solution is added which leads to colour change from blue to yellow. Then measurement is made photo metrically at 450 nm in an ELISA reader (Stefanovic et al.,2015).

The absorbance is inversely proportional to the aflatoxin concentration in the sample i.e., the lower the absorbance, the higher the aflatoxin concentration. The main instrument used in enzyme-linked immunosorbent assay is the ELISA reader. Itis basically a photometric instrument which gives the absorbance of the solution at the end of the process.(Hussain, 2011).

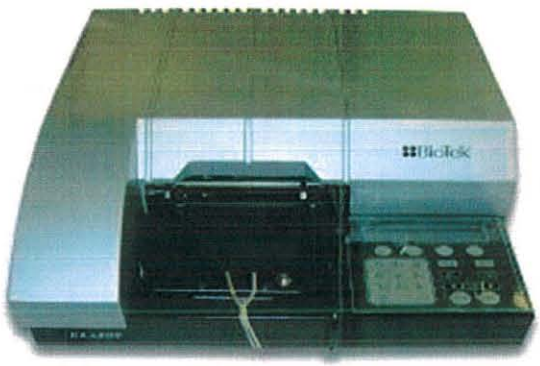

Figure 2.9: An ELISA reader

The ELISA reader gives absorbance readings from which % absorbance is calculated. For standard solutions, the % absorbance is plotted against aflatoxin concentration to get the calibration curve. The aflatoxin concentration is on x-axis and % absorbance is on y-axis. From the calibration curve, aflatoxin concentration is calculated for samples(Hussain, 2011).

ELISA has become the screening technique of choice for determination of mycotoxins in general, especially in situations where large number of samples is to be analyzed in a short period of time (Krska et al., 2005). However, one of the major drawbacks of immunoassays is the lack of information on the structure of the analyte (Stefanovic etal.,2015). Therefore in this research ELISA method is used because a large number of samples were analyzed, a short time period was given to finish the research and it doesnt need qualified personnel to conduct the analysis relative to the other methods.

Chapter 3

MATERIALS AND METHODS

3.1 Location of the study area

The study samples were collected from Bishoftu, a town and separate Woreda of Oromia regional state, Ethiopia. It is located 47.9 kilometres (29.8 mile) southeast of Addis Ababa and has an elevation of 1,920 meters (6,300 ft).

3.2 Sample collection and preparation

3.2.1 Sample collection

The samples were collected from three dairy industries by purchasing the products from their shops. Local butter and cheese (Ethiopian cottage cheese) samples were collected by purchasing each type of products from Bishoftu local markets. Local milk samples were taken directly from the farmers and local yogurt samples were purchased from different dairy shops. Collected samples were transported to the laboratory in refrigerated containers (4°C) and stored at -20°C until analysis according to Ghanem and Orfi (2009).

After observing the production schedule of the industries the samples were col-

lected every week to address different batch of samples. But in the case of local samples; all the samples were collected at once from different producers. For all local and industrial samples random sampling technique was used.

Table 3.1: *Sample collected from Bishoftu*

	Dairy type			
sample source	Milk(n)	Yogurt (n)	Cheese (n)	Butter (n)
Industrial	56	83	72	72
Local	52	10	10	10

3.2.2 Sample preparation for Milk

An aliquot of milk sample was removed from the refrigerator and 12ml was taken after bringing the sample to room temperature and centrifuged at 3,500rpm for 5 minutes to induce separation of the upper fatty layer. The upper fatty layer was removed by aspiration and the lower plasma was used in the assay.

3.2.3 Preparation for cheese sample

1g of finely grated or otherwise macerated cheese was mixed with 5mL of absolute methanol in a capped tube and mixed for 5 minutes. The tube was clarified by centrifugation (5,000g for 5 minutes) and the supernatant was removed. From this supernatant 0.5ml was transferred to a glass tube and the methanol evaporated by a steam of air (better recovery with nitrogen gas). Semi solid viscous material was deposited on the inside of the tube. After that 0.5ml of the provided blank skim milk was added to the tube and vortexed vigorously for one minute. The tube was allowed to stand for further 5 minutes and 100μl of this extract was used in the assay.

3.2.4 Sample preparation for yogurt sample

Sodium citrate extraction buffer at 7%concentration was prepared by adding 7gm of sodium citrate in to 100ml volumetric flask and make up to a final volume using distilled water. This solution was warmed to 50 °C in a water bath.

From this extraction buffer 5ml was taken and mixed with 5gm of yogurt sample. This mixture was placed in a water bath for 15minutes at 50°C and it was followed by centrifugation at 3,500 rpm (Revolution per minute) for 15min.The upper fatty layer was removed by aspiration and the lower plasma was used in the assay.

3.2.5 Sample preparation for butter samples

Methanol extraction buffer with 50% concentration was prepared by adding equal volumes of water and pure methanol (e.g. 2.5mL methanol + 2.5mL deionizer water). From this extraction buffer 5mLwas taken and 5gm of butter sample was added. The butter sample was placed at37°C in water bath and kept till it melts completely.

After the sample has melted completely it was mixed thoroughly for 1 minute and centrifuged for 5 minutes at 3,500 rpm. A fatty layer which was formed and solidified at the top of the extract was removed and the sample underneath the fatty layer was used for the assay.

3.3 ELISA Method of Analysis for AFM1

Determination of AFM1 in milk and dairy product was conducted using ELISA (enzyme-linked immunosorbent assay) method by HELICA Aflatoxin M1 Assay (USA) which is used for the quantitative detection of Aflatoxin M1 in milk and dairy products.

The HELICA Aflatoxin (Ml) Assay was a solid phase competitive enzyme immunoassay. An antibody with a high affinity for aflatoxin (Ml) was coated onto polystyrene micro wells. Standard or sample were added to the appropriate well and aflatoxin (Ml) present in the sample was bind to the coated antibody. Subsequently, aflatoxin bound to horse - radish peroxidase (HRP) was added and it was bind to the antibody which was not already occupied by aflatoxin (Ml) present in the sample or standard.

After the incubation period was completed, the contents of the wells were decanted, washed and an HRP substrate was added which develops a blue colour in the presence of an enzyme. The intensity of the colour was directly proportional to the amount of bound conjugate and inversely proportional to the amount of AFM1 in the standard or sample. Therefore, as the concentration of AFM1 in the sample or standard was increased, the intensity of the blue colour was decreased.

3.3.1 Determination of AFM1

All the reagents used for the analysis were obtained as a donation from Helica Bio systems, Inc (USA). After the reagents were received, all were immediately kept in a refrigerator (+4°C) until use. On each day of the analysis the reagents were removed from the refrigerator and bring to room temperature before use. PBS-Tween packet which was provided with the kit was reconstituted by washing out the contents with a gentle stream of distilled water into a 1 litter container and the solution was made up to 1 litter with distilled water and it was stored in refrigerator (+4°C) when not in use.

One mixing well was placed in a microwell holder for each standard and sample to be tested. An equal number of anti body coated microwells were also placed in another microwell holder. Unused wells were returned to the pouch and re-sealed to avoid the entry of moisture. The well holder was retained for future use. From sample diluents200μL (Micro litter) was dispensed into each mixing well and 100μL of standards and samples were dispensed (using a fresh pipette

tip for each) into the appropriate wells and mixed by aspirating three times. Using a multichannel pipette, 100μL of the mixtures were transferred to the corresponding antibody coated microtiter well and in cubated at room temperature for 10 minutes. Enough solution was in the mixing wells to run each standard and/or sample in duplicate if so desired. Sufficient conjugate (120μL per standard/sample) was placed in a trough and with a multichannel pipette 100L of conjugate was added to the wells already containing standard/sample. Wells were not washed before conjugate was added. Sufficient mixing was done by the force of the addition of the second 100μL to the first 100μL. Then it was incubated at room temperature for 30 minutes. Contents from microwells were decanted into a discard basin.

The microwells were washed by filling each with PBS-Tween wash buffer, which was prepared at the beginning of the analysis, and decanting the wash into a discard basin. Washing was repeated for a total of 3 washes. The required volume of substrate solution (1mL/strip or 120μL/well) was measure and placed in a separate container. Similarly100μLwas added to each microwell and incubated for 10 minutes. It was covered to avoid direct light.

The required volume of stop solution (1mL/strip or 120μL/well) was measured and placed in a separate container. From the stop solution 100μL was taken and added in the same sequence and at the same pace as the substrate solution was added. The optical density (OD) of each microwell was measured with a microliter plate reader using a 450nm filter. The optical density (OD) of each microwell was recorded. The zero standard was sated as 100% binding (Bo), binding% (%B) was calculated for each standard and sample as a percentage of the zero binding (%B/Bo).

3.4 Quality assurance and Quality control

The validity of the method was assured and controlled by determining within assay, between assay precision, accuracy, recovery and linearity results.

3.4.1 Between assay reproducibility

Precision of the method was evaluated through assay reproducibility; which was done by assaying three replicates of two different standards (M1 free Milk, 0.5ppb Standard, 0.4ppb Standard) in three different days The mean and coefficient of variation (CV) for the independent assay was calculated and evaluated.

3.4.2 With in assay reproducibility

Within assay reproducibility was evaluated by assaying three replicates of three different samples from each variety of dairy products (milk, yogurt, cheese and butter) during the same day, under the same experimental conditions and the mean and coefficient of variation (CV) for the three independent samples of each variety was calculated.

3.4.3 Accuracy

The accuracy of this analytically method was cheeked by assaying 0.5 *ppb* (Parts per billion) standard for eleven replicates of analysis and evaluating the mean value with the expected result(0.5 *ppb*) and this value was compared with the true value under.

3.4.4 Recovery

The extraction efficiency was evaluated by standard addition (spiking). A known amount of standard (0.4 *ppb*) was added to each variety of dairy products and a duplicate assay was conducted. The amount of sample taken for spiking from each variety was based on the assay procedure (12ml, 1gm, 5gm and 5g for milk, cheese, butter and yogurt samples respectively). The mean percentage recovery was calculated for each.

3.4.5 Linearity

Linearity between the percentage OD (%OD) and aflatoxin M1 concentration was studied by selecting six standards (0.0*ppb*, 0.1*ppb*, 0.25*ppb*, 0.5*ppb*, 1*ppb* and 2*ppb*) which were provided with the kit. A minimum of three standards were analyzed and a regression equation was found by plotting the %OD (y) versus the aflatoxin M1 concentration (x) expressed in ppb for every assay conducted and the R^2 was cheeked if it lies between 0 and 1, inclusive.

3.5 Calculation and Interpretation of Results

A dose-response curve using either the unmodified OD values or the OD values expressed as a percentage of the OD of the zero standards against the aflatoxin M1 content of the standard was constructed. Unknowns are measured by interpolation from the standard curve.

The mean value of the absorbance values obtained for the standards and the samples were divided by the absorbance value of the zero standards and multiplied by 100. The zero standard was made equal to 100% and the absorbance values of other standards and samples were quoted in percentages of this value.

$$\%Absorbance = \frac{Absorbancestandard(orsample)}{Absorbanceofzerostandard} * 100 \tag{3.1}$$

The above equation is Percentage absorbance calculation

The values calculated for the standards were entered in a system of coordinates on semi logarithmic graph paper against the aflatoxin M1 concentration in μg/L. The aflatoxin M1 concentration in μg/L corresponding to the absorbance of each sample was found from the calibration curve.

In order to obtain the aflatoxin M1 or concentration in μg/L actually contained in a sample, the concentration read from the calibration curve was further multi-

plied by the corresponding dilution factor. This is 1 for milk and butter samples 5 for cheese sample and 3 for yogurt sample which means, milk was not diluted, butter was diluted five times, and cheese was diluted three times.

3.6 Methodology for the study of knowledge, attitude and practice (KAP) towards aflatoxins contamination

A total of 41 personnel who were involved in the production process of the dairy products were selected for this study where 21 from the factories and 20 from the local producers (10 from milk producers and 10 from local market). These people were randomly selected from the list of factories targeted by the research and from local dairy producers where the sample was taken.

3.7 Statistical analysis

All ELISA readings ware conducted in duplicate and the data was averaged and expressed in the form of mean + standard deviation. One-way analysis of variance (ANOVA) and Multiple comparison tests were used to separate significantly different means; Significance was accepted at the probability $p<0.05$. All the analysis was carried out by using Statistical Product Service Solution (SPSS) version 21 software. Descriptive statistics and Microsoft excel ware also used to summarize the data.

Chapter 4

RESULT AND DISCUSSION

4.1 Quality control and assurance

4.1.1 Between assay reproductibility

Assay reproducibility was evaluated by assaying three replicates of two different standards (M1 free milk Standard and 0.4*ppb* Standard) and the mean and percentage coefficient of variation (%CV) for the independent assays was calculated. The results on the %CV values are as shown in the Table 4.1bellow and it was under the acceptable range according Helica (2016) it is acceptable if CV <15%.

4.1.2 with in assay reproducibility

Within assay reproducibility was evaluated by assaying three replicates of samples from each type of dairy products (milk, yogurt, cheese and butter) during the same day, under the same experimental conditions and the mean and % CV for samples of each dairy type was calculated. The % CV values are under the acceptable value according to Helica (2016) it is acceptable if CV < 10%.

4.1.3 Recovery

The extraction efficiency was evaluated by standard addition (spiking). A known amount of standard (0.4 *ppb*) was added to each type of dairy products and a duplicate assay was conducted. The amount of sample taken for spiking from each variety was based on the assay procedure (12ml,1gm,5gm and 5gm for milk, cheese, butter and yogurt samples, respectively). The mean percentage recovery was calculated as shown in annex and it is under the acceptable limit according to Maqbool et al. (2009) which is 70-110 % and according to Elgerbi et al. (2004) % recovery for milk is acceptable in a range between 66.85 to 98.25 %.

4.1.4 Accuracy

The accuracy of this analytical method was obtained by assaying 0.5 *ppb* standard for eleven replicates of analysis and evaluating the mean value with the expected result (0.5 *ppb*). And it was found out that the mean is not significantly different ($P < 0.05$) from the true value under study. The mean value found from the replicate analysis was annexed (Apendix III)

4.1.5 Linearity

In order to conduct a linearity relationship between the percentage OD (%OD) and aflatoxin M1 concentration; Linearity was studied by selecting six standards (0.0 *ppb*, 0.1 *ppb*, 0.25 *ppb*, 0.5 *ppb*, 1 *ppb* and 2 *ppb*) which were provided with the kit. A minimum of three standards were analysed and a regression equation was found by plotting the % OD (y) versus the aflatoxin M1 concentration (x) expressed in ppb for every assay conducted.

The value of regression coefficient (R^2) (Apendix VI) tells us that all the values of R^2 lies between 0 and 1, inclusive which is an indication of linear correlation between X and Y and the R^2 values are acceptable according to Maqbool et al., (2009).

4.2 Occurrence of aflatoxin M1 in milk and dairy products

For this study 108 milk, 83 Butter, 93 yogurt and 82 cheese samples were collected from dairy industries found in Bishoftu and the local market. A total of 440 samples were analyzed in duplicate including the quality control samples according to Helica (2016) assay procedure.

4.2.1 Occurrence of aflatoxin M1 in milk

Both samples from the local and industrial sources were collected for this analysis. From the total 108 milk samples 56 were from the industries and 52 were from the local producers.

Table 4.1: *Level of AFM1 in milk sample*

			Level of AFM1 in μg/L				
			Minimum	**Maximum**	**Mean**	**Std. Deviation**	**Range**
Sample type	N	Positive samples (%)	Minimum	Maximum	Mean	Std. Deviation	Range
Milk(Industrial)	56	100	0.550	1.41	0.970^{a}	0.212	0.911
Milk(Local)	52	100	0.029	2.159	0.690^{b}	0.505	2.123
Total	108	100	0.029	2.159	0.835	0.405	2.129

Values are mean, different superscripts in the same column represent statistically significant difference (P<0.05)

As shown in Table 4.1 above from 108 samples analysed for AFM1 all samples were found to be contaminated (100%) with a mean value of 0.835 μg/L. The highest AFM1 content was 2.159 μg/L and the lowest was 0.029 μg/L; both were obtained from the local milk producers.

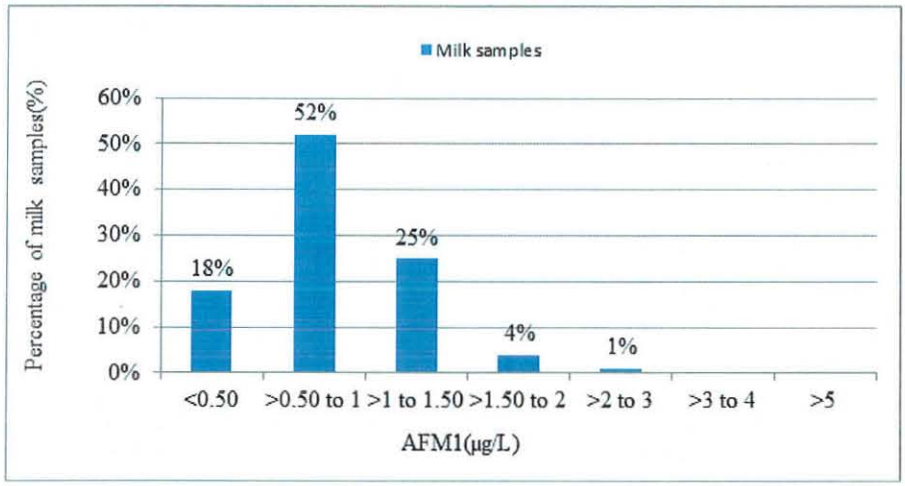

Figure 4.1: AFM1 distribution (N=108) and percentage of milk samples

In Figure 4.1 above the maximum percentage of samples (52%)was having AFM1 level between 0.50 μg/L and 1 μg/L.

As compared to the report from Gizachew et al. (2016), where only 9.10 % (n=10) of the samples were found in the range between 0.50 μg/L to 1 μg/L and the maximum percentage of samples (n=49) were having AFM1 level between 0.05 to 0.1 μg/L, our result was having the higher level of AFM1 contamination in almost half of the analysed samples from Bishoftu (Ethiopia).

And this shows the distribution of AFM1 level has increased in the area and requires serious considerations.

Table 4.2: *Comparison of AFM1 level in milk sample (N=108) with Ethiopian limit values*

	Ethiopia/US limit($\leq$ 0.5 μg/L)	
Milk sample	Under the limit	Over the limit
N	19	89
(%)	18	82

As shown in the Table 4.2 above, among the 108 samples analyzed only 19 samples (18%) are under the Ethiopian/Us acceptable limit value and 89 (82%) of the samples has failed to reach the limit value. As compared to Gizachew et al.

(2016), where only 19 sample were above Ethiopian/Us and 91 samples from 110 samples analyzed were within the acceptable limit value, our result was having the higher number or percentage of samples exceeding the Ethiopian/US limit. This shows that the occurrence of AFM1 has increased in the area as compared to the report in year 2015.

In this study 100% of the examined samples were having a positive result, which is similar to Alborzi et al.(2005) where from the 124 pasteurized milk samples collected from different supermarkets in Shiraz (south of Iran) during 6 months (April to September 2003) 100% of the samples were having a positive result. And 17.8% of the samples examined were having AFM1 level greater than the maximum tolerance limit (0.05 μg/L) accepted by European Union. Similar results were also observed by Gizachew et al. (2016) in Bishoftu Ethiopia, Kamkar et al (2010) in Iran, Ghazani (2009) in Tabriz (northwest of Iran); where all samples collected were contaminated by AFM1 and 91.8%, 26.3% and 26.3% exceed the EU limit, respectively.

On the other hand Kamkar (2005) from Iran reported a lesser percentage of positive samples (76.6%) having less mean level of AFM1 (0.118 μg/L) as compared to our result.

Similar results were also reported by Sadia et al. (2012) in Pakistan, Sassahara et al.(2005) in North of Parana state, Turkey Var and Kabak (2009) in Turkey, elik (2005) Turkey and Rahimi (2010) in Iran; having a percentage positive sample of 76.3% at 0.252 mg/L mean, 24%, 20% at 0.035 μg/kg mean, 88.23% and 42.1% at 43.3 0.044 μg/kg mean respectively. In contrast Ghanem and Orfi (2009) has reported a higher mean level of AFM1 (0.492 μg /kg) in pasteurized milk as compared to this study result but lesser value in raw cow milk (0.143 μg /kg).

From those reports it is observed that the occurrence of AFM1 has become a serious problem on human health especially in children because most infants use it daily in their diet. Also in this researchs report as mentioned above; from 108 samples collected 100% were positive for AFM1 with a mean of 0.835 μg/L, 82%

samples exceed the countries limit value and 52%were having AFM1 level between 0.50 μg/L and 1 μg/L; we can see that the prevalence of AFM1 in the area (Bishoftu, Ethiopia) is high. Which could also result from the contamination of the cattles feed by AFB1 due to improper handling and storage.

In the recent past, it has been indicated that many countries of Europe showed relatively low levels of contamination of AFM1 in milk and milk products. The occurrence of AFM1 at such low levels in European countries may be the result of stringent regulation of AFB1 in complementary feed stuffs for dairy cattle (Unusan, 2006). Since Ethiopia has already sated a limit value for AFM1 in milk, by in forcing the producers to produce the product at the acceptable limit and with contentious monitoring program by the responsible government body the country could have a chance to control the prevalence of AFM1 in the future.

4.2.2 Occurrence of aflatoxin M1 in yogurt

93 yogurt samples were collected for the analysis of AFM1 in yogurt and 10 were from local market and 83 were from the industrial source. As it can be seen in the Table 4.3 bellow all the analysed samples were found to be contaminated with AFM1 and the minimum level of AFM1 detected was 0.07 μg/kg and 4.76 μg/kg was the maximum, the mean level of AFM1 was 4.76 μg/Kg which is a high value.

Table 4.3: *Level of AFM1 in yogurt sample*

			Level of AFM1 in μg/L				
Sample type	N	Positive samples (%)	Minimum	Maximum	Mean	Std. Deviation	Range
Yogurt(Industrial)	83	100	0.09	4.01	1.631^a	1.04	3.92
Yogurt(Local)	10	100	0.07	4.76	1.628^b	1.70	4.70
Total	93	100	0.07	4.76	1.631	1.11	4.70

Values are mean, different superscripts in the same column represent statistically significant difference ($P<0.05$)

As shown in the Figure 4.3. bellow the maximum percentage of samples (27%)

was having AFM1 level between 0.50 μg/L and 1 μg/L, in addition, the second highest percentage of samples (24%) were having AFM1 level between 2 μg/L and 3 μg/L. which is a similar distribution to AFM1 level in milk. Since AFM1 level in yogurt has not been done in the country before we cant see the progress on the occurrence of AFM1.

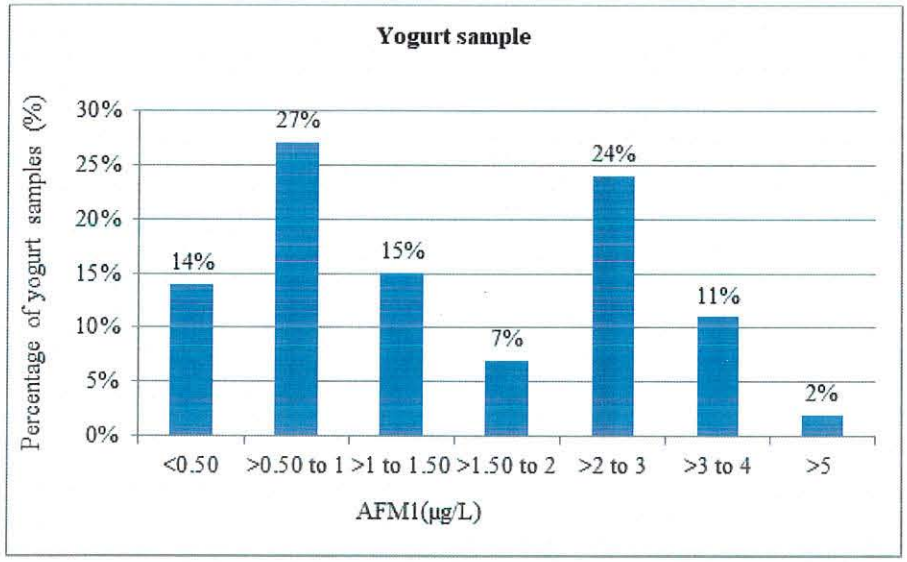

Figure 4.2: AFM1 distribution (N=93) and percentage of yogurt samples

Maqboolet al. (2016) reported that from 10 yogurt samples collected from 10 provinces of Pakistan, AFM1 was not detected in the four samples of Niab Colony, Bhaiwala, Saifabad and Waris Pura samples. Contaminated samples showed maximum concentration of 0.013 μg/Kgone in Samanabad samples and minimum concentration as 0.01 μg/Kg in Batala Colony and Peoples Colony. Having 0.05 μg /Kg as a limit value all the samples were below the acceptable limit which is under EU, Codex and Morocco regulations This value was very small as compared to our result from Bishoftu, Ethiopia.

A similar result was also found from a report by Iqbal and Asi (2013), where from 96 samples collected 61% were having a positive result on AFM1 with 0.147 + 0.017.5 μg/Kg as a mean value and 47 samples exceed EU limit. In addition, Fallah et al. (2010) reports that 66.1% yoghurt samples contaminated with AFM1 with a mean value of 0.032 μg/Kg but 20.6% of the samples collected were above

the acceptable limit sated by EU, Codex and Morocco (0.05 μg/Kg) which is a higher percentage as compared to Maqboolet al. (2016) but still a lesser percentage of samples as compared to our result from Bishoftu where 100% of the samples were above the acceptable limit value.

In general as compared to the above reports our result was having a higher percentage (100%) of samples exceeding the acceptable limit and with a higher mean level of AFM1 concentration (1.631 μg/Kg).This contamination of AFM1 could be from the contamination of milk sample used during yogurt production process. And it can be seen that the fermentation process during yogurt making doesnt have an impact on the level of AFM1.

Unlike cheese and milk samples, the presence of AFM1 in yogurt has not frequently been studied. Event there was no study on the occurrence of AFM1in yogurt from Bishoftu, Ethiopia to compare with our report Thus; more investigations are needed to set a limit value for the country and to control the occurrence of AFM1 in yogurt. Since currently, human consumption of yogurt has been greatly increased.

4.2.3 Occurrence of aflatoxin M1 in cheese

From 82 cheese sample collected 10 were from local and 72 were from industrial source .Table 4.5 bellow shows that all the analyzed samples (100%) were found to be contaminated with AFM1 with a mean value of 2.0 μg/Kg. The minimum concentration of AFM1 was 0.08 μg/kg from local source and 5.58 μg/kg as the maximum from industrial source.

Table 4.4: *Level of AFM1 in cheese sample*

			Level of AFM1 in μg/kg				
Sample type	N	Positive samples (%)	Minimum	Maximum	Mean	Std. Deviation	Range
Cheese (Industrial)	72	100	0.18	5.58	2.21^a	1.18	5.40
Cheese (Local)	10	100	0.08	3.86	0.77^b	1.24	3.78
Total	82	100	0.08	5.58	2.04	1.269	5.50

Values are mean, different superscripts in the same column represent statistically significant difference ($P<0.05$)

As shown in the Figure 4.3 bellow unlike the distribution graph from the other dairy types this time equal percentage distribution of samples at different concentration levels were observed. And the maximum percentage of samples (24%) lays both in AFM1 levels ranging from 1.50 to 2 μg/kg and from 2 to 3 μg/kg. This shows that around 48% of samples were having AFM1 level between 1.50 μg/kg to 3 μg/kg.

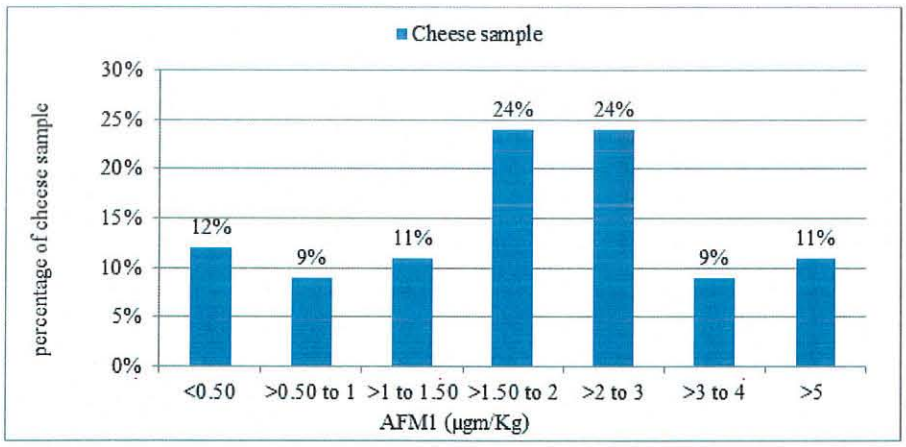

Figure 4.3: AFM1 distribution (N=82) and percentage of cheese samples

In general, from 82 samples collected from local and industrially produced cheese samples AFM1 was detected in all the samples (100%) with a mean value of 5.58 μg/Kg where 100% Exceed Egyptian, EU, Codex and Morocco regulations and 88% exceed US regulation. And the maximum percentage of samples (24%) lays both in AFM1 levels ranging from 1.50 to 2 μg/kg and from 2 to 3 μg/kg, which is 48% of samples were having AFM1 level between 1.50 μg/kg to 3 μg/kg.

According to Yitbarek and Tamir (2014), from the report on the level of AFM1 in cheese in the following countries Syria, Kazakhestan, Japan Spain, Turkey, Iran and Kuwaitno positive samples from Japan, Syria, Spain and few positive samples from Italy and Spain (4 and 16 respectively was found , whereas a high incidence of positive samples observed in Turkey that is 99 samples from 100 samples analyzed 32 samples from 40 in Kuwait and 66 from 80 samples in Iran. As regards the contamination level several authors found a high contamination level over 1.00 μg/Kg of AFM1 per kg but the maximum value among the seven countries was found in Turkey in 2005 which was 4.0 μg/Kg and this contamination level could be hazardous to human.

As compared to the above report the occurrence of AFM1 in cheese from Bishoftu (Ethiopia) is at higher level both on the number of positive samples that is 82 positive samples from 82 samples analyzed and having a maximum reported concentration of AFM1 that is 5.58 μg/kg, which is a high risk to human health.

4.2.4 Occurrence of aflatoxin M1 in butter

From 83 Butter sample collected 72 from shops of the 3 industries found in Bishoftu and10 were from local. Table 4.7 bellow shows from a total of 83 samples 78 (94%) of them were positive samples with a mean AFM1 value of 0.245 μg /Kg. The 1.24 μg/kg as the maximum from industrial source and minimum concentration of AFM1 was 0.0 μg/kg.

Table 4.5: *Level of AFM1 in butter sample*

			Level of AFM1 in μg/kg(*ppb*)				
Sample type	N	Positive samples (%)	Minimum	Maximum	Mean	Std. Deviation	Range
Butter (Industrial)	72	99	0.00	1.24	0.261^a	0.16	1.24
Butter (Local)	6	60	0.00	0.91	0.133^b	0.28	0.91
Total	78	94	0.00	1.24	0.245	0.18	1.24

Values are mean, different superscripts in the same column represent statistically significant difference ($P<0.05$)

A different AFM1 distribution was observed in the case of butter samples. As it can be seen in the Figure 4.4 bellow the maximum percentage of samples (95%) were having AFM1 level below 0.50 μg/kg and there were no samples with AFM1 level between 1.5 and 5 μg/kg; which is a better AFM1 distribution relative to the other dairy products.

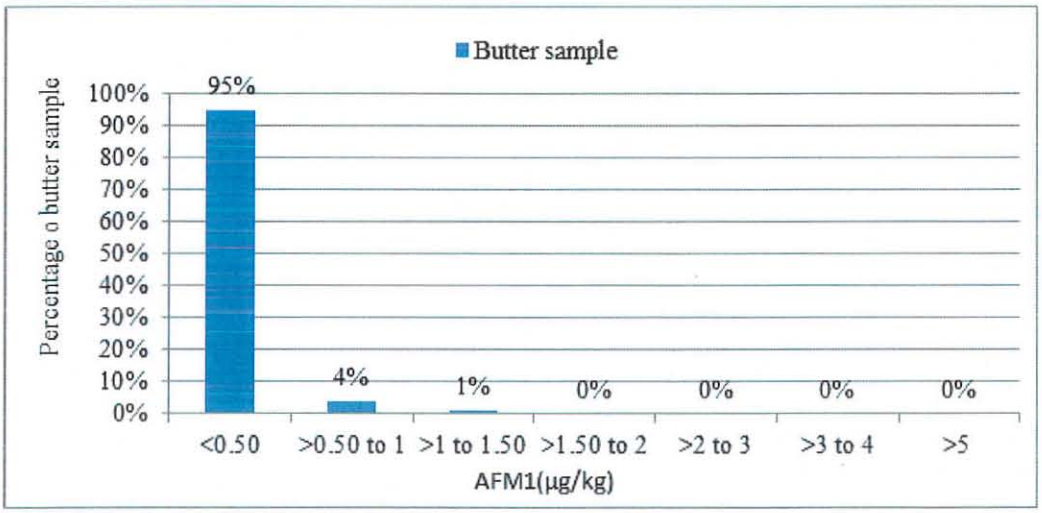

Figure 4.4: AFM1 distribution (N=83) and percentage of butter samples

According to Aksoy et al. (2016), report on the level of AFM1 by different researches conducted in Turkey, on a research conducted between September 2007 and September 2009 on 80 butter samples obtained from supermarkets in Erzurum, Turkey, by Aydemir Atasever it was reported that 66 samples were contaminated with AFM1 ranging from 0.01 to 0.12 μg/kg and 13 samples exceeded the maximum legal limit. In addition, Maqbool and M. Ahmad (2009) reported an extremely low level of AFM1 (non-detectable) with a maximum concentration of 0.074 μg/kg. Similarly Fallah (2010) reported that from 31 butter samples collected 8 (25.8%) were positive samples with mean concentration of 0.005+0.002 μg/kg and a maximum and minimum level of AFM1 of 0.015 μg/kg and 0.132 μg/kg, respectively.

Therefore, our result has a similarity with a report from Maqbool and M. Ahmad (2009) by having low levels of AFM1 in the result. But the mean level of AFM1

found in our result was still having a higher level of AFM1 as compared to a report Maqbool and M. Ahmad (2009) and other reports (Aksoy et al. 2016;Fallah ,2010) which could result from the primarily contamination of row milk which was used as a row material for butter production . Since this level of AFM1 found shows a high prevalence of AFM1 in yogurt in the specified area (Bishoftu, Ethiopia); it should be regulated from the source (row milk) through good agricultural, feeding and storage practices.

4.2.5 Comparison of AFM1 level in yogurt cheese and butter with limit values

As compared to different acceptable limit, Table 4.6: bellow shows thatall positive yogurt samples were exceeding the Egyptian, EU, Codex and Morocco regulations. In the case of US regulations (0.5μ g/kg) only 13(14%) of the samples were under the limit and more percentage (86%)of the samples were exceeding the US regulation. Also all positive cheese samples were exceeding the Egyptian, EU, Codex and Morocco regulations. In the case of US regulations (0.5μg/kg) only 10(12%) of the samples were under the limit and more percentage (88%) of the samples were exceeding the US regulation.

Table 4.6: *Comparison of AFM1 level in yogurt, cheese and butter with limit values*

		Limit values of AFM1 in μg/L					
		EU/Codex/Morocco limit(< 0.05μg/L)		**US limit(< 0.5μg/L)**		**Egypt/limit(< 0μg/L)**	
sample		Under the limit	Over the limit	Under the limit	Over the limit	Under the limit	Over the limit
Yogurt	N	0	93	13	80	0	93
	(%)	0	100	14	86	0	100
Cheese	N	0	82	10	72	0	82
	(%)	0	100	12	88	0	100
Butter	N	8	75	79	4	5	78
	(%)	10	90	95	5	6	94

In the case of butter from 94%(78) out of the 83 samples collected 75(0%) of posi-

tive butter samples were exceeding the EU, Codex and Morocco regulations. But in this dairy product type 79(95%) were under the acceptable limit of US regulations (0.5 μgm/kg) and only 4(5%) of the samples were exceeding the limit. In the case of Egyptian regulation 5(6%) samples can meet the regulation this time. In addition 6% samples were found to have zero level of concentration (acceptable limit of Egyptian regulation) and 95% were under the acceptable limit of US regulations, but still 90% of the sample exceed EU, Codex and Morocco regulations. In addition, even if there are samples with zero level of AFM1 having a mean level of 0.245 μg/Kg is still a higher value.

4.3 Variation on the level of AFM1 between the four dairy types

From the analytical result found by analyzing dairy products (Milk, yogurt, cheese and butter) different level of AFM1 was observed between the four types of dairy products.The following Table 4.9 shows the amount of samples analysed for each types of dairy products and their minimum, maximum and mean value of aflatoxin M1 detected in *ppb* (μg/kg). As shown in the Table 4.9 the highest mean value of aflatoxin M1 was found in cheese (2.036 μg/kg)t hen followed by yogurt, milk and butter sample based on an ascending order of their mean level of aflatoxin M1.

Similarly, from the number of samples analysed the highest value of aflatoxin was detected also in the cheese sample which was 5.58 μg/kg followed by yogurt with 4.76 μg/kg, milk 2.16 μg/kg and butter sample 1.24 μg/kg based on a descending order on the level of AFM1. In addition to this while observing the mean concentration of all the four types, still cheese was the one with highest mean concentration of AFM1 (2.04 μg/kg) followed by yogurt with 1.63 μg/kg, milk 0.83 μg/kg and butter sample 0.24 μg/kg.

Table 4.7: *Level of AFM1 level between dairy types*

	Level of AFM1 in μg/kg(μg/L)					
Type of sample	N	Minimum	Maximum	Mean	Std. Deviation	Range
milk sample	108	0.03	2.16	0.83[a]	0.40538	2.13
yogurt sample	93	0.07	4.76	1.63[b]	1.11568	4.70
cheese sample	82	0.08	5.58	2.04[c]	1.26876	5.50
Butter sample	83	0.00	1.24	0.24[d]	0.18573	1.24

Values are mean, different superscripts in the same column represent statistically significant difference (P<0.05)

Our result has shown some variation with a report by Iqbal and Asi (2013) report where the mean concentration of AFM1 in milk yogurt cheese and butter were 0.212+11.9 μg/kg, 0.147.1 +17.5 μg/kg , 0.189 +34.7 μg/kg and 0.156+ 21.4 μg/kg,respectively and the maximum mean level of AFM1was found in Milk, followed by Cheese, butter and yogurt and a report from Maqbool and Ahmad (2009) report in Pakistan, where from the 3 dairy products analysed the maximum was found in Milk 0.040 μg/ L followed by Yogurt maximum concentration of 0.013 μg/ L and Butter samples showed extremely low levels of AFM1 (non-detectable).In our result the maximum AFM1 level was in cheese and the minimum in butter.

Our result shows similarity with a report from Fallah (2010) pasteurized milk samples (mean: 0.052 μg/kg; range: 0.0130.250 μg/kg), 45 (66.1%) yoghurt samples (mean: 0.032 μg/kg; range: 0.0150.119 μg/kg), 59 (81.9%) white cheese samples (mean: 0.297 μg/kg; range: 0.0301.200 μg/kg), 8 (25.8%) butter samples(mean: 0.005 lg/kg; range: 0.0130.026 μg/kg) and the maximum AFM1 concentration was frond in cheese followed by milk, yogurt and butter. But still AFM1 level in milk was having a higher value than yogurt.

Different researches were made on the effect of various dairy product manufacturing processes on the level of AFM1. According to Oruc et al. (2006), even though the distribution of aflatoxin M1 between whey and the final cheese varies depending on the type of cheese being made; during cheese production process,

due to the high affinity of AFM1 to the casein and its semi polar characteristics, the higher percentage of AFM1 present in raw milk had passed to curd (52% & 58%) than whey (47% & 40%). Aksoy et al. (2016) also showed that 75% of M1 found in the curd and 25% occurred in the whey. Similarly, (Sengun et al., 2008; Kaniou-Grigoradou et al., 2005; Kamkaret al, 2008) reports ranging between 66-80% the greatest proportion of AFM1was found in the curd.

As discussed above in the research report highest value of AFM1 found in our result could also be due to the binding of AFM1 (Aflatoxin M1) to milk proteins a more toxin going into the curd than into the whey. In addition to this the susceptibility of mold growth in cheese is higher during storage the increase of AF could also be due to the growth of aflatoxigenic molds on these products.

On the other hand the lowest value of AFM1 in the butter sample could be, as already been mentioned above a more portion of AFM1 was moved to the cured therefore small ratio of AFM1 in milk was carried-over to cream, and yet a smaller proportion to butter according to Aksoy et al. 2016, sinceit binds more to the pretentious part of the milk than fat portion (butter) a lower level of AFM1 was detected.A similar argument with a research report from Prandini et al., 2009and Bakirci, 2001 was given.

In addition to this as mentioned by Bakirci (2001) the highest value of AFM1in yogurt sample relative to milk could be due to the extraction efficiency of the method to yogurt than milk. Our result was having difference with the report from Govaris et al. (2002) who found a significant decrease of AFM1 levels in all yoghurt samples from those initially present in milk. Therefore, in our research result the decrease in AFM1 levels due to factors during fermentation process such as low pH, formation of organic acids or other fermentation by-products, or even presence of lactic acid bacteria was not observed.

Similarly, in our report the effect of pasteurization on the decrease of AFM1 level was not observed. According to Govaris et al. (2002), this result could be due to the stability of AFM1 in milk during pasteurization, sterilization or freezing. But

his idea contrasts with a report from Deveci(2007) where a significant decrease on the level of AFM1 (12.4% and 9.1% respectively) after pasteurization of two milk samples having 4.9 μg and 3.5 μg of AFM1 at 72°C for 2min was observed.

In general, while comparing the level of AFM1 concentration in all the dairy types with the above research reports, our result shows a higher level of AFM1 concentration in milk and milk products, since it is a threat to public health and it should be continuously monitored.

4.4 KAP

4.4.1 General information

In this study a total of 41 respondents were selected and 57.5 % of the respondents were employees in some dairy industries in Bishoftu area where the samples were collected and 42.5% were from the local milk suppliers and dairy product producers. All the respondents believe that drinking milk and consuming dairy products is good for health. About their educational background only 12.8% from the total respondents were degree or diploma holders. In addition, 74.5%of the respondents drink or consume at least one type of dairy product per day.

4.4.2 Knowledge, attitude, and practices towards aflatoxins contamination

4.4.2.1 KAP response from the local producers

From Table 4.10 it is observed that majority of the respondents (100% with mean score of 3) had knowledge about mold and they also knew that feeding animals with a feed contaminated by a mold could contaminate their milk and they knew the conditions that could favour its formation. But 75% of the respondents had no idea about aflatoxin with a mean score of 1.5.

In general, according to Azaman et al. (2016), having a mean score of 2.25 local producers had an adequate knowledge to aflatoxin related questions which shows that the respondents in this study do not have adequate knowledge about the health impact of aflatoxin.

Table 4.8: *Local producers knowledge on aflatoxin contamination*

Food Safety Knowledge towards Aflatoxins Contamination	Respond%			Mean
Statement	1	2	3	
1. Do you have any idea about aflatoxin?	75	0	25	1.5
2. Did you ever observed mold in any of the animals' feed?	0	0	100	3
3. Do you know that animal feed/dairy product with mold can be contaminated with aflatoxin?	75	0	25	1.5
4. Do you know feeding animals with a feed contaminated with mold could contaminate their milk?	0	0	100	3
5. Do you know that intake of aflatoxin in milk and dairy products have adverse health implications?	75	0	25	1.5
6. Do you know conditions that favor mold formation on animals feed?	0	0	10	3

Note 1=I have no idea, 2=I am not sure, 3=Yes

Table 4.11 shows that 100% of the respondents agreed that feed having mold need to be separated and discarded properly and also believe that rodents and insects can facilitate mold formation on feeds during storage. Therefore, it is possible to conclude that local producers have favourable attitude towards aflatoxin contamination even though they lack the knowledge of the health impact of aflatoxin. In addition to this, 85% of them never had given a feed having mold to a cattle but 15% of them did give a feed having mold which could contaminate the cattle milk with aflatoxin if it is from Aspergillussource. All of the respondents were not sure whether a safe product from aflatoxin can be judged by seeing or tasting the product. It could be because they dont have much knowledge about aflatoxin they cant give answer weather it can solely be judged by sight or not.

Table 4.9: *Local producers Attitude towards aflatoxin contamination*

Food Safety Attitude towards Aflatoxins Contamination	Respond%			Mean
Statement	1	2	3	
1. Did you ever feed cattle with a feed covered with mold?(Disagree=3,nutral =2 and Agree=1,)	85	0	15	2.7
2. Do you agree with the idea that entrance of rodents and insects facilitate mold formation on feeds during storage?	0	0	100	3
3. Do you agree that a safe product from aflatoxin can be solely judged by sight and taste?	0	100	0	2
4. Do you separately put feeds having mold and discard it properly	0	0	100	3

Note:1=Disagree, 2= Neutral and 3=Agree

As shown in the Table 4:12 bellow, even though 100% of the respondents having a maximum mean score of 3 said that they always clean their production area, the utensils and equipment they used after work; the overall mean score on hygienic practices for the local producers is 1.97, indicating that most of respondents observed low practices in terms of hygiene and sanitation towards reduction of aflatoxins contamination.

The reason for this low practice is because 100% of the respondents with a minimum mean score of one had responded that they have never conducted any quality test for their products, never checked the quality of the feed before receiving and they never had a program to regularly disinfect the premises.

Table 4.10: *Local producers hygienic practices towards aflatoxin contamination*

Food Safety Knowledge and Hygienic Practices towards Aflatoxins Contamination	Respond%			Mean
Statement	1	2	3	
1. Do you frequently clean your production area?	0	0	100	3
2. Do you always wash your hands (with soap and water) and dry before entering to the	0	15	85	2.85
3. Do you clean utensils and equipment after work?	0	0	100	3
4. Do you check the quality of the feed before receiving?	100	0	0	1
5. Do you have a program to regularly disinfect the premises?	100	0	0	1
6. Do you conduct any quality test for your products?	100	0	0	1

Note:1=Never,2= Seldom and 3= Always

4.4.2.2 KAP response from employees in dairy industries

The Table 4.13 bellow contains the result on the response of dairy industry employees towards a question asked on their knowledge about aflatoxin contamination. The table shows that unlike the respondents from the local producers at least 22.2 % of the respondents have knowledge about aflatoxin but still 66.7% had no idea and 11.1% were not sure about their knowledge, therefore the mean score on their general awareness about the toxin found to be 1.56.

The lowest mean score which is 1.26 was observed in a statement, Do you know using raw milk contaminated with aflatoxin could contaminate the other forms of dairy products, where 81.5 % of the respondents had no idea about it.

The next low mean score that is 1.44 was from two statements, Do you know that animal feed/dairy product with mold can be contaminated with aflatoxin and Do you know that intake of aflatoxin in milk and dairy products have adverse health implications Where majority of the respondents (77.8%) had no idea about it. But similar to the local producers 100% of the respondents had high score of 3 on knowledge question about conditions which favour mold formation on animal feed/dairy product. And 100% of respondents respond to the statement Did you ever observed mold in any of the animals' feed/ products with the highest mean score that is 3.

In general from the seven questions asked having a mean score of 1.947 the employees in dairy industries have inadequate knowledge towards aflatoxin contamination.

Table 4.11: *Knowledge about aflatoxin contamination on Employees of dairy industries*

Food Safety Knowledge towards Aflatoxins Contamination	Respond%			Mean
Statement	1	2	3	
1. Do you have any idea about aflatoxin?	66.7	11.1	22.2	1.56
2. Did you ever observed mold in any of the animals' feed/ products?	0	0	100	3
3. Do you know that animal feed/dairy product with mold can be contaminated with aflatoxin?	77.8	0	22.2	1.44
4. Do you know that feeding animals with a feed contaminated by mold could contaminate their milk?	48.1	11.10	40.7	1.93
5. Do you know that intake of aflatoxin in milk and dairy products have adverse health implications?	77.8	0.00	22.2	1.44
6. Do you know what conditions favour mold formation on animal feed/dairy product?	0	0	100	3
7. Do you know using row milk contaminated with aflatoxin could	81.5	11.1	7.4	1.22

Note:1=I have no idea, 2= I am not sure and 3=Yes

The response on an attitudes question towards aflatoxin by dairy industry employees are shown in the Table 4.14 bellow. Among the four questions asked 100% of employees agreed to a statement Do you separately collect defective products and discard them properly? with a maximum mean score of 3.

Next to this a 2.519 mean score was found from a statement Do you cheek if the package is free from any contaminants before packing where 48.1% of the respondent were neutral and 51.9% of them has agreed on the statement. The types of packages used in dairy industries are film for milk, cup with aluminium and transparent plastic bag for cheese packing.

To a statement Do you agree that a safe product from aflatoxin can be solely judged by sight and taste Most of the respondents (55%) has disagreed with a mean score of 2.22. The smallest mean score found is in a statement Do you agree with the idea that entrance of rodents and insects facilitate aflatoxin contamination of feeds/dairy product during storage? that is 1.778 where 48.1% of them has disagreed with the idea and the rest of the respondents has a greed and act neutral equally to this statement.

The overall mean score for this attitude question that is 2.38 shows that they have

positive attitude towards the toxin according to Azaman et al. (2016).

Table 4.12: *Attitude towards aflatoxin contamination on Employees in dairy industries*

Food Safety Attitude towards Aflatoxins Contamination	Respond%			Mean
Statement	1	2	3	
1. Do you cheek if the package is free from any contaminants before packing?	0.0	48.1	51.9	2.519
2. Do you agree with the idea that entrance of rodents and insects facilitate aflatoxin contamination of feeds/dairy product during storage?	48.1	25.9	25.9	1.778
3. Do you agree that a safe product from aflatoxin can be solely judged by sight and taste?	55.6	40.7	3.7	2.220
4. Do you separately collect defective products and discard them properly?	0.0	0.0	100	3.0

Note:1=Disagree, 2=Neutral and 3= Agree

As shown in Table 4.15 below among the 9 statements majority of the respondents (100%) had always practiced the four statements (statements 1, 3,4and5) with a maximum mean score of 3.
This implies that in the dairy industries production areas are frequently cleaned, all the utensils and equipment are kept cleaned in proper place after work and they also have a program to regularly disinfect the premises. Some of the factories mount an electric lamp to block the entrance of flies and insects in to the production area.

Almost half of the respondents (59.3%) always practice the activities in two statements Do you conduct any quality test for your product and Do you check the storage temperature With a mean score of 2.481 and 2.37, respectively. Even though the score 2.481 is the second highest mean score the quality tests performed by the factories are PH test during yogurt production which is not directly related to aflatoxin test.

Only 14.8% of the respondents always practice the statement Do you always wash your hands (with soap and water) and dry before entering to the production area with a mean score of 2.148.

The lowest mean score was observed in the statements Does your company have

awareness creation program on Aflatoxin And Do you taste your products for Aflatoxin which are1.74 and 1.37 respectively and this statements are not always practiced by the factories where the respondents are employed. And this mean values are even found because of some of the respondents present in a factory where an aflatoxin test was conducted once and this factory has planned to continue on doing it in the near future.

In addition to this an awareness creation program is also on its way to be implemented properly with schedule on giving awareness for the row milk suppliers once in three months by this factory too.

The overall mean score for the hygienic practice towards aflatoxin by employees in dairy industries was observed to be 2.46. According to Azaman et al. (2016), this result indicates that most of respondents observed high hygienic and sanitation practices towards reduction of aflatoxins contamination in dairy products despite that fact that the sample analysis showed significant deviation from acceptable aflatoxin M1 ranges.

Table 4.13: *Hygienic practices towards aflatoxin contamination on employees in dairy industries*

Hygienic Practices towards Aflatoxin contamination	Respond%			Mean
Statement	1	2	3	
1. Do you always wash your hands (with soap and water) and dry before entering to the production area?	0.0	0.0	100	3.00
2. Did you ever observed mold in any of the animals' feed/ products?	0.0	85.2	14.8	2.148
3. Do you clean utensils and equipment after working and keep in a proper place?	0.0	0.0	100	3.00
4. Do you check the quality of the feed /row milk before receiving?	0.0	0.0	100	3.00
5. Do you have a program to regularly disinfect the premises?	0.0	0.0	100	3.00
6. Do you conduct any quality test for your products?	11.1	29.6	59.3	2.481
7. Do you taste your products for Aflatoxin?	63	37	0.0	1.37
8. Do you check the storage temperature?	22.2	18.5	59.3	2.37
9. Does your company have awareness creation program on Aflatoxin?	7.4	92.6	0.0	1.74

Note:1=Never, 2=Seldom and 3=Always

Table 4.14: *Summary mean scores of knowledge, attitude, and practices between respondents.*

	Sample mean score of respondents	
Level	Dairy industry employees	Local producer's
Knowledge	1.950	2.250
Attitude	2.380	2.670
Practices	2.460	1.975

From the Table 4.16 it is observed that dairy industries are having a knowledge gap by having a minimum mean score of 1.950 and local producers had a gap on practices with mean score of 1.975 towards aflatoxin contamination.

And from the total score of respondents the maximum is found from the local producers under the attitude questions that is 2.670 this result agreeswith Bas et al. (2006) who pointed out that improved knowledge will encourage positive attitudes and safety behaviors among the food handlers.

The second highest mean score was found from the dairy industry employees at the practice question with the value of 2. 460. This shows that even though most of the employees had no adequate knowledge specific to the toxin the factory they are employed had a positive practice towards minimizing aflatoxin contamination. This practice is developed through few (22%) of the employees in the factory with a high level of education and an adequate level of knowledge on aflatoxin contamination.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Among 366 samples collected from Bishoftu area 244 and 361samples were above the acceptable limit sated by US standard ($\leq 0.5\mu g/kg$) and Egyptian standard ($0\mu g/kg$) respectively. Also according to EU standard ($\leq 0.05g/kg$) from 258 samples (Yogurt, cheese and butter) collected 250 samples has exceeded the limit. This value appears to be very high and a big concern for milk and dairy products in Bishoftu market.

In addition to this, the maximum value of aflatoxin among all the analyzed samples was found in cheese which was $5.580+0.08\mu g/kg$ and the minimum in butter sample. From these values we can conclude that the different separation takes place during the production process of butter and cheese makes affect AFM1 concentration.

While looking in to the mean concentration of AFM1 in yogurt from both sources (Local and industrial) and milk raw (local) and pasteurized (Industrial), the effect of pasteurization and fermentation process on AFM1 level was not observed.

Under comparing the occurrence of aflatoxin between the local and industrial sample sources, all dairy types from local source were having lower mean level of AFM1 contamination relative to the samples from industrial sources. The maximum mean concentration of aflatoxin in the industrial dairy products may be due to pre-concentration and cross contamination during the production process.

On the other hand on the KAP study even though the local producers have knowledge on mycotoxin related questions their knowledge specific to AFM1 is not adequate. In addition to this, their practice towards reducing the contamination level of AFM1 is not adequate enough. But they have a positive attitude towards reduction of AFM1 level.

Unlike the local producers employees in dairy industry had a better practice towards reducing the level of AFM1 contamination, but they still dont have adequate knowledge about the health impact of aflatoxin.

5.2 Recommendation

- Since milk and dairy products are highly consumed in the country and AFM1 is a carcinogenic chemical it recommended that laws and regulations need to be set to control the quality of dairy products and establishing a limit value to the three dairy products (cheese, yogurt and butter) by the responsible authorities.
- It is recommended that mitigation measures and regular awareness creation programs are set and implemented to control aflatoxin contamination by the concerned ministries.
- It is recommended that all of the samples are further analyzed using LC-MS-MS to accurately substantiate the ELISA study and take serious measures to reduce aflatoxin contamination before it creates significant health impacts on consumers.
- All the dairy industries should work in collaboration with the row milk suppliers under the implementation of good food hygienic practices towards AFM1.
- Even though researches have been done about level of AFM1 in milk so far, this study shows that the problem on the occurrence of the toxin still persists. Therefore, as it is practiced in different food products the government should establish programs to randomly take samples from the market and cheek for AFM1 levels regularly and take corrective mitigation measures.

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Appendix A

Standard AFM1 calibration curves for different assay

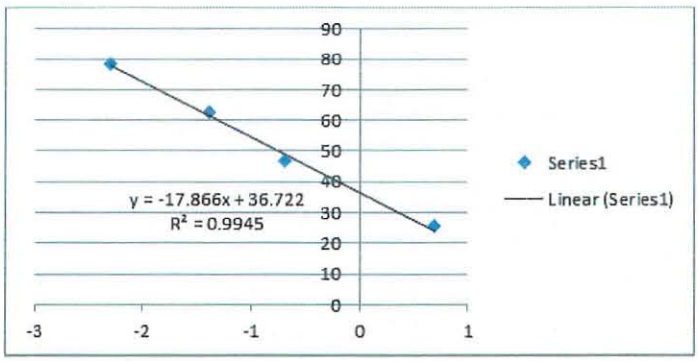

Figure A.1: Assay 1

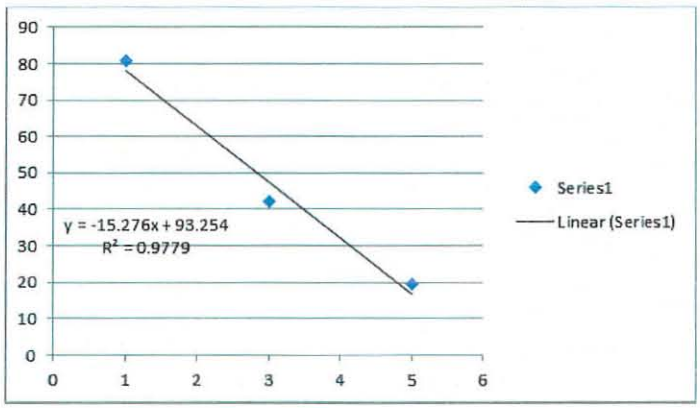

Figure A.2: Assay 2

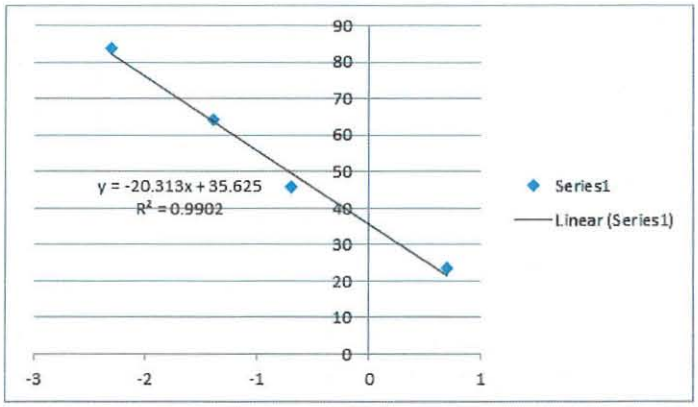

Figure A.3: Assay 3

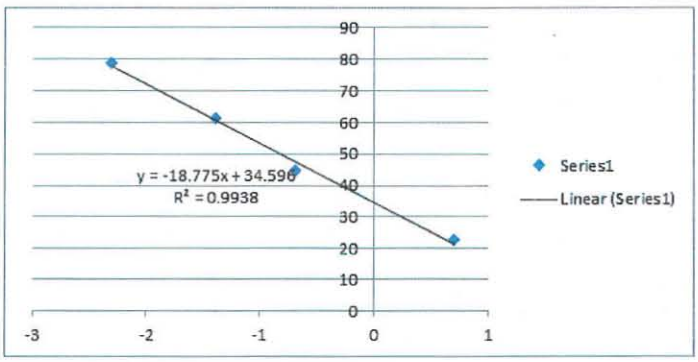

Figure A.4: Assay 4

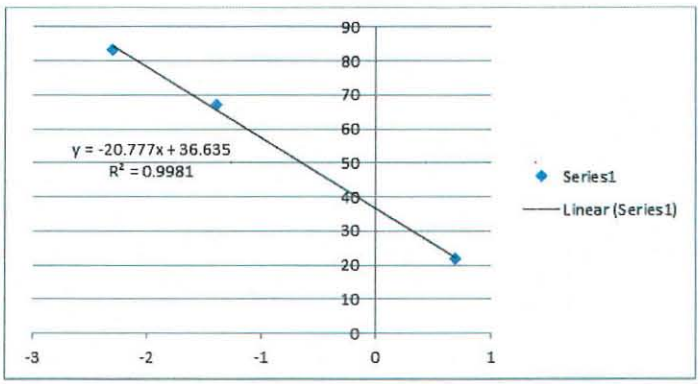

Figure A.5: Assay 5

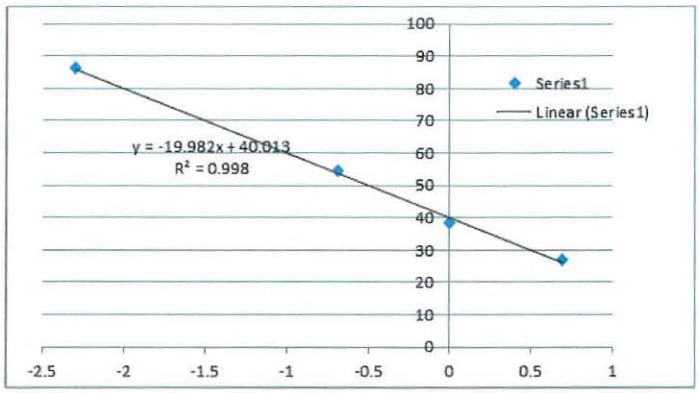

Figure A.6: Assay 6

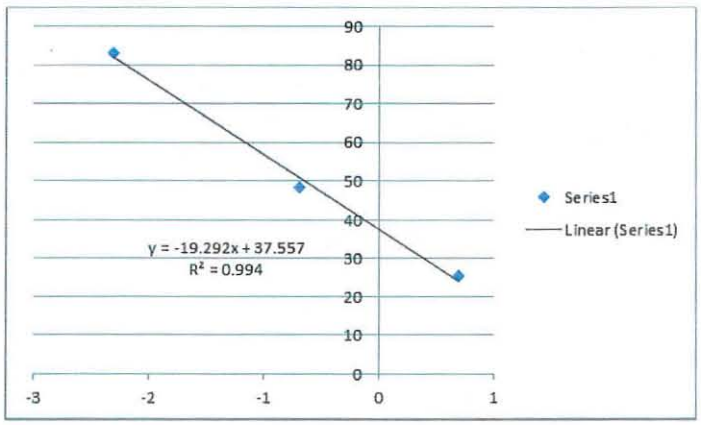

Figure A.7: Assay 7

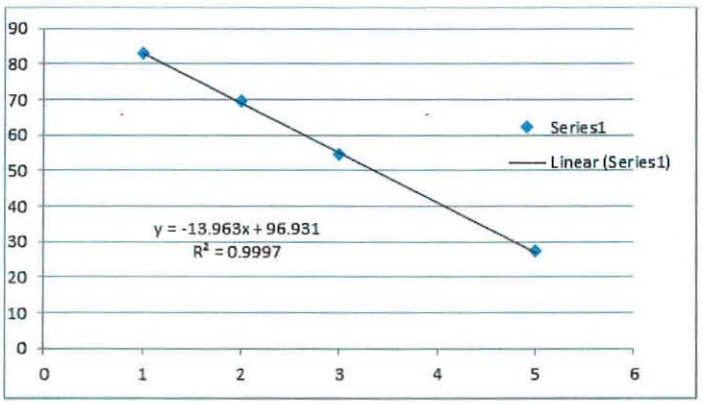

Figure A.8: Assay 8

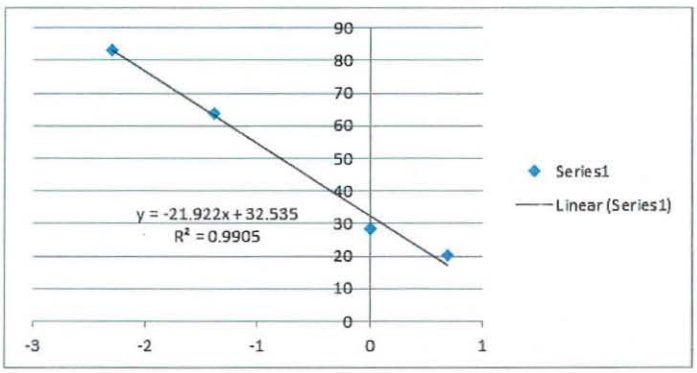

Figure A.9: Assay 9

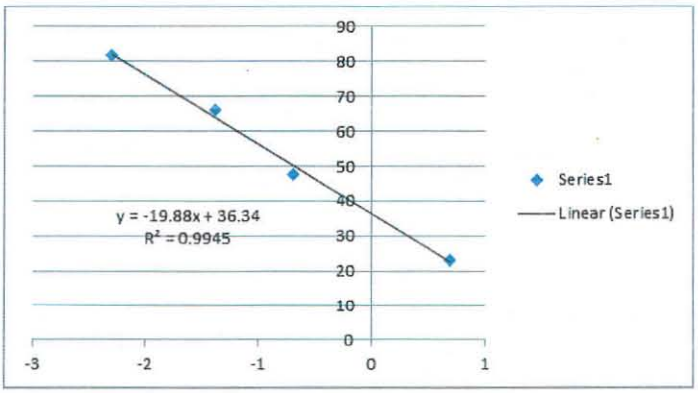

Figure A.10: Assay 10

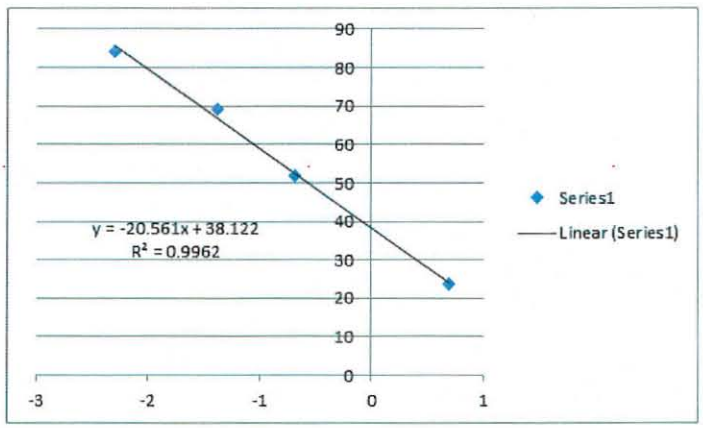

Figure A.11: Assay 11

Appendix B

Between assay reproducibility

Table B.1: *Between assay reproducibility*

	Assay 1	Assay 2	Assay 3	Avg *ppb*	CV Between Assay	%CV Between Assay
M1free milk	0.02	0.03	0.03	0.03	0.13	13
0.4*ppb* Standard	0.36	0.36	0.44	0.39	0.11	11

Appendix C

Within assay reproducibility

Table C.1: *Within assay reproducibility*

	Replicate 1	Replicate 2	Replicate 3	Avg *ppb*	CV with in Assay	%CV with in Assay
Milk	0.87	0.97	0.91	0.92	0.05	5.00
Yogurt	0.86	0.96	0.95	0.92	0.01	1.00
Cheese	0.84	0.96	0.91	0.90	0.04	4.00
Butter	0.13	0.14	0.16	0.10	0.1	1.00

Appendix D

Assay recovery calculation

Table D.1: *Assay recovery calculation*

	Initial Sample reading	Spiked concentration *ppb*	Spiked sample reading	%Recovery	Average %recovery
Milk	0.92	0.40	0.97	73.54	77.19
	0.92	0.40	1.07	80.03	
yogurt	0.92	0.40	1.48	111.72	107.57
	0.92	0.40	1.37	103.41	
cheese	0.91	0.40	1.32	101.15	101.15
Butter	0.14	0.40	0.4	73.33	72.02
	0.14	0.40	0.38	70.71	

Appendix E

Mean value for replicate analysis of 0.5 *ppb* standard

Table E.1: *Mean value for replicate analysis of 0.5 ppb standard*

	Assay 1	**Assay 2**	**Assay 3**	**Assay 4**	**Assay 5**	**Assay 6**	**Assay 7**	**Assay 8**	**Assay 9**	**Assay 10**	**Assay 11**	**Mean** *ppb*
0.5*ppb* Standard	0.76	0.42	0.58	0.51	0.52	0.57	0.41	0.65	0.54	0.41	0.56	0.54

Appendix F

Linearity

Table F.1: *Linearity*

Assay	Calibration curve equation	R^2
1	$17.866X + 36.722$	0.9945
2	$15.276X + 93.254$	0.9779
3	$20.561X + 38.122$	0.9962
4	$19.88X + 36.34$	0.9945
5	$21.922X + 32.535$	0.9905
6	$13.963X + 96.931$	0.9997
7	$19.292X + 37.557$	0.9940
8	$19.982X + 40.013$	0.9980
9	$20.777X + 36.635$	0.9981
10	$18.775X + 34.596$	0.9938
11	$20.313X + 35.625$	0.9902

Appendix G

Questionnaire for KPA analysis on aflatoxin

G.1 Questionnaire for industries

Questionnaire for KPA analysis on aflatoxin

A) Questionnaire for industries

Dear respondent

Good morning/Good afternoon. Thank you for your interest to take this interview with me today. I am SELAMAWIT TADESSE who is a post graduate student of Addis Ababa University center for food science and nutrition, conducting a study to determine the prevalence of aflatoxin in milk and dairy products in *Beshoftu area.* 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. Your honest answers to these questions will help me for a better understanding of the topic, and will eventually help in designing and implementing appropriate interventions to alleviate related problems.

I greatly appreciate your participation in the study. I want to inform you that any information collected in this research will be utilized only for the research objectives.

Date: _____

Are You willing to take the interview **Yes** ☐ **No** ☐

1. personal Information

Name (if you are willing to give your name)

Address:

Telephone number:

Job Title

Educational background

Role in Dairy industry Employee ☐ Producer ☐

Questions	**Yes**	**NO**	**I have no idea**	**Comment**
2. General Information Questions				
1. Do you believe that drinking milk and consuming dairy products is good for health?				
2. Do you consume at least one type				

2. Do you consume at least one type of dairy?				
3. Food Safety Knowledge towards aflatoxin Contamination				
1. Do you have any idea about aflatoxin?				
2. Did you ever observed mold in any of the animals' feed/product?				
3. Do you know that animal feed/dairy product with mold can be contaminated with aflatoxin?				
4. Do you know that intake of aflatoxin in milk and dairy products have adverse health implications?				
5. Do you know that intake of aflatoxin in milk and dairy products have adverse health implication?				
6. Do you know that conditions favor mold formation on animal feed/dairy product?				
7. Do you know using row milk contaminated with aflatoxin could contaminate the other forms of dairy products?				
4.Food Safety Attitude towards aflatoxin Contamination				
1. What type of packaging do you use for the packaging of dairy product?	-	-	-	**List the packaging material here**

2. Do you cheek if the package is free from any contaminants before packaging?				
3. Do you agree with the idea that iterance of rodents and insects facilitate aflatoxin contamination of feed/dairy product during storage?				
4. Do you agree that a safe product from aflatoxin can be solely judged by sight and taste?				
5. Do you separately collect defective products and discard them properly?				
5. Hygiene Practices towards aflatoxin Contamination				
1. Do you frequently clean your production area?				
2. Do you always wash your hands (with soap and water) before entering to the production area?				
3. Do you clean utensils and equipment after working and keep in a proper place?				
4. Do you check the quality of the feed/row milk before receiving?				
5. Do you have a program to regularly disinfect the premises?				
6. Do you conduct any quality test for your products?				

7. Do you cheek the storage temperature?				
8. Does Your company have any awareness creation program on spread of aflatoxin?				

Do you have any comments or suggestions for this research? _____

_____

_____

_____

_____

_____

_____

THANK YOU!!!

G.2 Questionnaire for local producers

B) Questionnaire for local producers

Dear respondent

Good morning/Good afternoon. Thank you for your interest to take this interview with me today. I am SELAMAWIT TADESSE who is a post graduate student of Addis Ababa University center for food science and nutrition, conducting a study to determine the prevalence of aflatoxin in milk and dairy products in *Beshoftu area.* 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. Your honest answers to these questions will help me for a better understanding of the topic, and will eventually help in designing and implementing appropriate interventions to alleviate related problems.

I greatly appreciate your participation in the study. I want to inform you that any information collected in this research will be utilized only for the research objectives.

Date: _____

Are You willing to take the interview **Yes** ☐ **No** ☐

1. personal Information

Name (if you are willing to give your name)

Address:

Telephone number:

Job Title

Educational background

Role in Dairy industry Employee ☐ Producer ☐

Questions	**Yes**	**NO**	**I have no idea**	**Comment**
2. General Information Questions				
1. Do you believe that drinking milk				

during storage?				
4. Do you separately put feeds having mold and discard it properly?				
5. Hygiene Practices towards aflatoxin Contamination				
1. Do you frequently clean your production area?				
2. Do you always wash your hands (with soap and water) before entering to the production area?				
3. Do you clean utensils and equipment after working and keep in a proper place?				
4. Do you check the quality of the feed before receiving?				
5. Do you have a program to regularly disinfect the premises?				
6. Do you conduct any quality test for your products?				

Do you have any comments or suggestions for this research? _____

_____

_____

_____

_____

_____

_____

THANK YOU!!!