Thesis Reference No. _____

ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

STUDY ON THE LEVEL OF AFLATOXIN M1 IN RAW COW MILK FROM DAIRY FARMS AND COLLECTION CENTERS IN AND AROUND ADAMA TOWN

MVSC THESIS

BY

TAYE SOLOMON ABEGAZ (ID GCR/4163/14)

JUNE, 2023

BISHOFTU, ETHIOPIA

ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

STUDY ON THE LEVEL OF AFLATOXIN M1 IN RAW COW MILK FROM DAIRY FARMS AND COLLECTION CENTERS IN AND AROUND ADAMA TOWN

BY

TAYE SOLOMON ABEGAZ

ADVISOR

BIRUHTESFA ASRADE

(DVM, MSc, ASSOC. PROFESSOR)

CO-ADVISOR

GEZAHEGN MAMO

(DVM, MVSc, PhD, PROFESSOR)

A thesis submitted to the College of Veterinary Medicine and Agriculture, Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Veterinary Science in Veterinary public Health MVSc in Veterinary Public Health

JUNE, 2023

BISHOFTU, ETHIOPIA

ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY PUBLIC HEALTH AND AGRICULTURE

DEPARTMENT OF IMMUNOLOGY,MICROBIOLOGY

AND VETEREINARY PUBLIC HEALTH

STUDY ON THE LEVEL OF AFLATOXIN M1 IN RAW COW MILK FROM FARMS AND COLLECTION CENTERS IN AND AROUND ADAMA TOWN, EAST SHEWA,CENTRAL ETHIOPIA

Submitted by: Taye Solomon Abegaz _____ _____

Signature Date

Approved for submittal to the dissertation assessment committee

1. Dr. Biruhtesfa Asrade (DVM, MSc, Assoc.Professor) _____ _____

Advisor Signature Date

2. Gezahegn Mamo (DVM, MVSc, PhD, Professor) _____ _____

Co- advisor Signature Date

3. prof. Bekele Megersa_ _____ _____

Department chairperson Signature Date

APPROVAL

ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY PUBLIC HEALTH AND AGRICULTURE

As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by: Taye Solomon Abegaz entitled '**Study on the Level of Aflatoxin M1 in Raw Cow Milk From Dairy Farms and Collection Centers in and Around Adama Town, Central Ethiopia**' and recommend that it be accepted as fulfilling the thesis requirement for the degree of Masters of Veterinary Science in Veterinary Public Health.

1. Dr. Geremew	_____	_____
Chairman	Signature	Date
2. Dr. Ketema Tafes	_____	_____
External Examiner	Signature	Date
3. Dr. Fanos Taddese	_____	_____
Internal Examiner	Signature	Date
4. prof. Bekele Megersa	_____	_____
Department chairperson	Signature	Date

STATEMENT OF THE AUTHOR

I affirm that this research is solely my own work, and that all sources of materials used in this thesis work have been properly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for Master of Veterinary Science in Veterinary Public Health degree at Addis Ababa University's College of Veterinary Medicine and Agriculture, and it has been deposited in the College library to be made available to borrowers in accordance with the Library's rules. I solemnly declare that I am not submitting my thesis to any other institution anywhere for the award of any academic reward. Brief quotations from this thesis are allowable without special permission provided that acknowledgement of source is appropriately made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however permission must be obtained from the author.

Name: Taye Solomon Abegaz : Signature _____
College of Veterinary Medicine, Bishoftu, Ethiopia
Date of Submission: 16/ 06/2023

ACKNOWLEDGEMENT

I want to start by thanking the All-Powerful God. I want to express my gratitude to my primary advisor. Thanks to Dr. Biruhtesfa Asrade (Associate Professor) and Prof. Gezahegn Mamo, College of Veterinary Medicine and Agriculture, Addis Ababa University, for his general research guidance, revision of the thesis, helpful suggestions, and patience in helping to shape this study work from the beginning to the end.

I would also like to convey my gratitude and admiration to the Ethiopian Agricultural Authority's Animal Product, Veterinary Drug, and Feed Quality Assessment Center and all of its staff members working in the toxicology team (Dr Bedaso Kebede, Dr. Husen Bedu, Mr. Fantu Fekadu and Dr. Ermias Gebeyehu) for providing me with all essential materials and technical assistance. My deepest gratitude is extended to Dr. Belachew Bacha, for his unwavering efforts, helpful criticism, and technical and material assistance for this research project from its inception to its completion. His recommendations and consistent follow-up contributed to the success of this study.

My heartfelt gratitude goes to the students in my batch especially Veterinary public health department students (Ageri, Misge, Segni, Miki, Wondu, Teferi, Melke and Geme) for their collaboration throughout our education and research period, which enabled us to successfully complete the entire program. I also value their courtesy and desire to perform what is needed of us during our time at college. Finally, I wish them all the best in the world as they face them. I won't soon forget. Teachers at the College (especially MVPH) for the immeasurable time they invested in teaching their students and imparting their wisdom so we might succeed in our coursework and research.

Finally yet importantly, I would want to thank my family for all of the help they have provided me throughout my career. I appreciate the patience my wife Salam, my two young daughters Gabreilla and Yanet, Lelo and the third child on the way have showed me over the course of my professional career. I really apologize for taking my children's time away from you to enjoy this with me.

DEDICATION

This thesis is dedicated to my wonderful deeply missed mom on October 1/2013. Forever you remain in my soul.

“I thought of you with love today,
But that is nothing new,
I thought about you yesterday,
and days before that too.
I think of you in silence,
I often speak your name.
All I have are memories,
and your pictures in a frame.
Your memory is my keepsake,
With which I will never part.
God has you in his keeping,
I have you in my heart”

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFB1	Aflatoxin B1
AFG1	Aflatoxin G1
AFM1	Aflatoxin M1
AFM2	Aflatoxin M2
AFs	Aflatoxin
AOAC	Association of Analyitcal Chemists
APIC	Animal Products and Inputs Quality Testing Center
CSA	Central Statistics Agency
ELISA	Enzyme Linked Immuno Sorbent Assay
ESA	Ethiopian Standard Agency
EU	European Union
FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
FLD	Fluorescent Detector
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
LC	Liquid Chromatography
TLC	Thin Layer Chromatography
USA	United States of America
USAID	United States Aid Development
UV	Ultra violet
VDFACA	Veterinary Drug and Animal Feed Administration and Control Authority
WHO	World Health Organization

LIST OF ANNEXES

ABSTRACT

Livestock is vital to the livelihood of people and to the economics of many developing countries like Ethiopia. Besides to this, animals are the main source of protein particularly for human diets. The presence of a carcinogenic toxin like Aflatoxin (AF) animal products poses a serious health risk. The aim of this study was to detect and quantify the level of aflatoxin M1 (AFM1) in raw cow milk collected from dairy farms and collection centers in and around Adama town. A cross-sectional study was conducted from December 2022 to April 2023 on 120 raw cow milk samples with the Association of Analyitcal Chemists (AOAC) official method of analysis using reverse phase high performance liquid chromatography (HPLC). A questionnaire survey was employed as a tool to assess dairy producers' Knowledge, Attitude and Practice (KAPs) towards aflatoxins (AFs). While determination of AFM1 in milk was performed by liquid chromatography technique. 100 ml of raw cow milk samples were collected from dairy farms and milk collection centers using a falcon tube for determination of AFM1. Samples were cleaned up with immunoaffinity column and assaying of AFM1 was carried out using HPLC with a fluorescent detector and post column derivatization technique. The Knowledge, Attitudes and Practice (KAP) findings depicted that 75.8% of respondents have concepts about mold growth and toxin formation, and 76.8% know about favorable conditions for mold growth. From the total of 120 milk samples, 73 (60.8%) were contaminated with AFM1 from which 9.2% were found to have aflatoxin level above the Ethiopian Standards (ES) regulatory limit, and 34.7% were above the European Union acceptable limit. Binary logistic regression was used to see the association between predictor and outcome variables. Logistic regression analysis results showed that farm management practices like moisture control, feed source, storage place, and time were significantly associated with the occurrence of AFM1 in milk. The level of contaminated milk with AFM1 reported in this study should be a wakeup call for stringent monitoring of raw materials and feed samples to prevent cattle exposure to AFs contaminated feed which would lead to excretion of AFM1 in milk and eventually causing human exposure through consumption of contaminated milk.

Keywords: Aflatoxin, milk, dairy farm, Adama, HPLC

1. INTRODUCTION

Livestock is vital to the livelihood of people and to the economics of many developing countries like Ethiopia. Besides to this, animals are the main source of protein particularly for human diets. Moreover, consumption of livestock and livestock products in Ethiopia is increasing due to the increased level of livelihood and income of the people. Milk and milk products are important sources of different nutrients mainly like calcium and phosphorus. These are generally the main dietary requirements for human being mainly both mothers and infants. Consequently, the contamination of these products by a carcinogenic toxin like aflatoxins is a great health concern. Therefore, milk and milk products have to be inspected and controlled continuously for aflatoxin contamination (Sani and Nikpooyan 2013).

There is numerous human health risks associated with milk due to presence of AFM1. Farmers and traders have no significant knowledge about the health risks related to long term exposure to AFs (Firew *et al.*, 2020). Aflatoxin M1 (AFM1) has a potency which is close to that of the potency of aflatoxin B1 (AFB1). Therefore, it is important to determine Aflatoxin M1 levels in milk to protect children and adult from its potential health hazards. AFM1 is resistant to autoclaving, pasteurization and thermal inactivation. Aflatoxin M1 has both acute and chronic effect (Sani, Nikpooyan 2013). Aflatoxin M1 is classified as group one causative agent of human cancer by International Agency for Research on Cancer (IARC) (Sharma *et al.,* 2019). The occurrence of AFs in human, animal and milk products is one of the most serious health problem of food safety since milk is important food for adults, and the unique nutrient for infant (Polak-Śliwińska, 2020). Aflatoxins are significantly more of an issue in the tropics than in temperate zones. However, due to the global movement of agricultural commodities, no region of the world is free of AFs (IARC, 2002).

Aflatoxins are toxic by-products of *Aspergillus,* a fungus that lives in the soil and is in charge of decomposing plant matter (Bankole and Adebanjo, 2003). Twenty AFs, including Aflatoxin B1 (AFB1), Aflatoxin B2 (AFB2), Aflatoxin G1 (AFG1), Aflatoxin G2 (AFG2), Aflatoxin M1 (AFM1) and Aflatoxin M2 (AFM2), are known to occur. The letters B and M stand for the colors blue and green respectively, under florescence detection. The equivalent metabolites of AFB1 and AFB2 are AFM1 and AFM2, respectively. According to Jaimez *et al*. (2000),

AFM1 and AFM2 present in foods derived from animals, such as milk and milk derivatives, meat, and eggs. According to the International Agency for Research on Cancer (IARC), AFB1 is the most harmful and carcinogenic of them all and is a class 1 human carcinogen (IARC, 1993).

Following the ingestion of AFs contaminated feed by lactating dairy cows AFMI is secreted into the milk. Aflatoxin M1 is a major metabolite of parent AFB1 that is formed by enzymatic hydroxylation at the 9a-position and has an approximate overall conversion rate equal to 1 to 3%. Consequently, exposure to AFs can occur directly through contaminated foods or indirectly through AFMI contaminated milk. Excretion of AFM1 in milk can take 12–24 hours after the ingestion of AFB1, but there is a decrease in concentration after 72 hours (Bukari *et al., 2020*). Therefore, it is important to determine AFM1 levels in milk to protect the public especially infants and olds from its potential health hazard (Karimi *et al.,* 2020).

According to Firew *et al.*, (2020), the production value chain in Ethiopia is highly vulnerable to AF contamination. The production of AFs can start in the field throughout the crop growing cycle and continue during harvesting, drying, processing, and storage steps, strongly depending on various environmental conditions (Pereira *et al.*, 2019). Firew *et al.* (2020), reported about one milk processing farm in Ethiopia that was forced to dispose of 129,000 liters of milk produced because of AFM1 contamination. In 2015, in Ethiopia, the International Livestock Research Institute (IRLI) published the results of its survey on AFs in Addis Ababa area, and 93% of milk samples collected were contaminated with AFM1, which was significantly higher than allowed maximum residue limits (MRLs) in the European Union (EU) (0.05µg/l). Thus, the detection of high AFs levels in oilseed cakes and compound feeds has raised serious concerns about ensuring the desired quality and safety of feed along the food value chain (Seyoum *et al.,* 2018).

On the other hand, information on AF contamination of feed in Ethiopia is very limited, confined to limited market samples, and does not particularly address the situation at production (Ephrem *et al.,* 2015). Dawit *et al.,* (2016) revealed that the AFB1 contamination levels of the feed collected from different actors along the value chain were fairly similar,

indicating that the contamination may occur early on. In a similar way factors that determine the risk of mycotoxin contamination of oil seed cakes are more likely to be associated with earlier stages of the production and distribution chain. Accordingly, some literature recommends future studies in Ethiopia to identify the points at the oil seed cake production and distribution chain where mycotoxin production risk factors are prevalent and the potential risk mitigation opportunities (Merwe *et al.,* 2019).

Many nations have imposed limits on its content in feed and foods because of its hazardous health effects. The United States standard, which is set by the Food and Drug Administration (USFDA), indicates that it should not be higher than 0.5ug/l in liquid milk (Merwe *et al.,* 2019). The Commission regulation of the European Union (EU) sets that the maximum level of AFM1 in liquid milk should not exceed 0.05ug/l

Aflatoxin contamination of milk is one of the important issues that require proper attention in order to protect the public. Previous investigation conducted in the Addis Ababa milk shade area revealed high levels of AFM1 contamination in milk, which at the time was a major national concern and raised public awareness of AF (Dawit *et al.,* 2016). Therefore, the objective of this study is to detect and quantify the level of AFM1 in raw cow milk collected from dairy farms and collection centers in and around Adama town. Additionally questionnaire survey was conducted to assess Knowledge, Attitude and Practice (KAP) of dairy farm owners about mold growth and formation of toxin.

1.1. Significance of the Study

The result of this study will be useful in figuring out how much milk in Adama town is contaminated with AFM1. It can also be used to assure the safety of milk products for human consumption as well as to protect the health of dairy farmers, feed producers, and consumers raising awareness of AF contamination in milk, milk products, and animal feed, The information gathered through this study will be used as the foundation for determining the Ethiopia’s AF regulation limits. The study's findings can be used as a resource by academics and researchers in the subject. In general, the findings of this study will strengthen Ethiopia's efforts to build a safe food and feed industry.

1.2. Research Questions

1. What is the level of aflatoxin M1 in milk in the study sites, in and around Adama town?
2. What are the knowledge, attitude and practice (KAP) of dairy farmers about aflatoxin?

1.3. Objectives

1.3.1. General objectives

- To detect and quantify AFM1 level in raw cow milk, to compare the results with the national and international standards and to collect information about knowledge, attitude and practice of dairy farmers on Aflatoxins in feed and milk.

1.3.2. Specific objectives

- To detect and quantify the level of AFM1 in raw cow milk and to evaluate or compare the result with national and international standards.
- To assess the Knowledge Attitude and Practice of milk producers about AF in milk and feed.

2. LITERATURE REVIEW

2.1. Mycotoxins

The term "mycotoxin" was first used in the 1960s to describe a toxin connected to turkey-x disease in England and tainted peanuts in animal feed. Later, it was discovered that this mycotoxin was *Aspergillus flavus* toxin, AFB1. When introduced in small amounts naturally to higher vertebrates and other animals, mycotoxins, which are toxic secondary metabolites generated by many species of microscopic filamentous fungi prevalent on field cereals, including barley, cause a hazardous reaction (Bennett *et'al.*,2003). Animal toxicity is just as dangerous as that of the fungi that produce these compounds. In addition to being acutely toxic, some mycotoxins are now linked to the development of specific types of cancer, raising concerns about the safety of feed and food worldwide, notably for milk and eggs (Bennett *et'al.*,2003).

Mycotoxins are secondary metabolites of fungus with very low molecular weight that are toxic to both human and animals. They are produced by a variety of fungi that can infect a wide range of agricultural items intended for human consumption and animal feed (Nogaim, 2014). Mycotoxins are common pollutants in raw materials, food, and feeds that are caused by mold species that may grow on a variety of substrates and in a variety of environmental settings. They are present in agricultural products all over the world. According to estimates, 25% of the world's crops are impacted by fungal growth, and goods may be tainted with mycotoxins both before and after harvest. Regarding the expected levels of mycotoxins in food, different countries have different expectations (Bennett and Klich, 2003).

Mycotoxins can be ingested, inhaled, or absorbed through the skin and exposure to them is almost usually unintentional. The majority of mycotoxicoses cases are caused by consuming contaminated food and exposure to humans can occur either directly through cereals or indirectly through animal products such as meat, milk, and eggs (Ames, 2003). The majority of mycotoxins is reasonably heat stable within the typical food processing temperature range (80-121°C), therefore routine cooking methods like boiling and frying or even after pasteurization cause little to no degradation. In general, mycotoxin concentrations are greatly

reduced but not entirely eradicated by the use of food processing, which includes physical treatments (cleaning and milling) and thermal processing (cooking, baking, frying, roasting, and extrusion). Mycotoxin concentrations are affected by various treatments in diverse ways, although those that use the highest temperatures have the biggest impacts. Roasting or boiling at high temperatures (over 150 °C) seems to dramatically lower mycotoxin concentrations (Bullerman and Bianchini, 2007).

2.2. Toxicity of Mycotoxin

When mycotoxins are ingested, inhaled, or absorbed through the skin, they can be dangerous to people and animals in low amounts. According to (Ilunga, 2012), the consumption of tainted food or feed is the primary way that both humans and animals are exposed to mycotoxin. Mycotoxicoses have been defined as food or feed related, noncontagious, nontransferable, and noninfectious, despite the fact that they are the most dangerous and create negative consequences when consumed directly from contaminated food products and foodstuffs (Zain, 2011). Depending on the species, mycotoxins affect humans and animals in a variety of acute and chronic ways. Age, sex, weight, food, exposure to infectious agents, the presence of other mycotoxins, and the presence of pharmacologically active compounds all affect how mycotoxins affect a certain species' health (Milicevic *et al.,* 2010). According to their hazardous activity under long term settings, the majority of known mycotoxins are classified as mutagenic, carcinogenic, or teratogenic (Niessen, 2007).

2.3. Epidemiological Factors for Mycototoxin Production

The production of mycotoxins is highly susceptible to temperature, moisture, water activity, pH and oxygen concentration, the same environmental factors that affect the growth of toxigenic fungi. Moisture and temperature are two factors that have a crucial effect on fungal proliferation and toxin biosynthesis (Bryden, 2007; Palumbo, 2010).

The incidence and level of mycotoxin contamination are closely related to the geographic position and to seasonal factors as well as to the cultivation, harvesting, stocking and transport conditions (Milicevic *et al.,* 2010).

Mycotoxin contaminations can be categorized into those that occur in the developing crop, or pre-harvest, and those that develop after maturation, or post-harvest. In the pre-harvest period, preventive measures are incorporated into good agronomic practices, such as the careful use of insecticides and fungicides, irrigation to avoid moisture stress, harvesting at maturity, and improvement by genetic resistance to fungal attack. Throughout the post-harvest period, the control of the moisture and temperature of the stored commodity will largely determine the degree of fungal activity and, consequently, the mycotoxin synthesis (Bryden, 2007).

The incidence of AFs in food and feed is relatively high in tropical and subtropical regions, where climatic conditions favor the growth of molds (Liu and Wu 2010). These areas have humid and dry climates that are highly suitable for the proliferation of fungal species, particularly *Aspergillus flavus* and *Aspergillus parasiticus*, which are the main producers of AFB1. Food grains may be contaminated by AFB1 at a number of stages, but it is most common when crops are exposed during harvesting and storage, provided that hot and humid weather conditions and improper and unsanitary storage exist for a prolonged period (Stepman, 2018).

The main predisposing factor in postharvest AF accumulation in food is poor storage conditions, namely excessive heat and moisture, pest-related crop damage, and extensive periods of time spent in storage (exceeding several months). Even commodities dried to a satisfactory degree before storage can develop local pockets favorable to AF growth as a result of moisture generated by insect respiration and local condensation. Genotypes, drought, soil types, and insect activity are important in determining the likelihood of pre-harvest contamination. (Mutegi *et al*., (2018).

Aflatoxin related disorders may manifest differently depending on animal species, age, diet, sex, and potential exposure to other toxins. Age and sex of the animals also affect their sensitivity to AFB1. For instance, the effects of AF are more likely to affect men than females, and young animals of all species are more likely to experience them than mature animals. Nutritional deficiencies, particularly those in vitamin E and protein, make people more

susceptible to AFs (Bryden, 2011).

Chemical treatments with substances such sodium bisulfite, ozone, ammonia, acids, and bases offer a chance to inhibit microbial development and the production of mycotoxin in grains that have been stored (Bozoglu, 2009). In recent years, effective mycotoxigenic fungal management has been attained by employing plant materials as environmentally friendly fungicides, such as extracts and essential oils (Reddy *et al.,* 2010; Thembo *et al*., 2010). Similarly to that, biological control gives a fresh chance for control methods. According to research by Bianchini and Bullerman (2010), lactic acid bacteria (LAB), propionic acid bacteria, and Bacillus species can all suppress the growth of mycoses and the generation of mycotoxin.

2.4. Aflatoxins

Aflatoxins are a group of related fungal secondary metabolites primarily produced by the fungi, *Aspergillus flavus* and *Aspergillus parasiticus*. *Aspergillus flavus* and *Aspergillus parasiticus* colonize a wide variety of food commodities including maize, oilseeds, spices, groundnuts, tree nuts, milk, peanut and dried fruits. However, production of AF by these fungi depends on drought stress, rainfall, suitability of crop genotype for its climate, insect damage, agricultural practices and postharvest conditions like storage, transportation and food processing (Wu *et al*., 2011).

A member of the mycotoxins family, AF is produced by *Aspergillus* species like *Aspergillus flavus* and *Aspergillus parasiticus.* In agriculture, *Aspergillus flavus* is extremely common. *Aspergillus bombycis*, *Aspergillus ochraceoroseus*, *Aspergillus nomius*, and *Aspergillus pseudotamari* are all AF producing species, despite seeing them much less commonly (Bennett and Klich, 2003). *Aspergillus nomius* and *Aspergillus parasiticus* both create AFB and AFG toxin however *Aspergillus flavus* and *Aspergillus parasiticus* are the primary manufacturers of AFs in agricultural products. *Aspergillus flavus* primarily produces AFB1. Aflatoxin B1 and AFB2 for blue fluorescence and AFG1 and AFG2 for green fluorescence, respectively, are the classifications for AFs based on fluorescence color. The metabolites are called milk AFs, commonly known as AFM1 and AFM2 (Wu *et al*., 2011).

2.5. Aflatoxin B1

The most prevalent of the four AFs described above, AFB1 has been discovered in cereals, feeds, oilseeds, and pulp (Buszewska-Forajta, 2020). It is the most dangerous strain, and a link to adverse health effects including liver cancer has been established. It is regarded as a human class I carcinogen, in accordance with USAID and Danya (2012). Demissie (2018,) asserts that the outcomes for animals rely on a variety of factors, including the quantity, length, species, breed, diet, and nutritional state. Anorexia, decreased milk output, lethargy, liver damage, and abdominal edema are the hallmarks of the toxicity experienced by dairy calves after consuming AFBI contaminated feed. Additionally, AFBl causes immune system suppression, which can lead to sickness and even death. High levels of AFB1 in people have been linked to vomiting, cramping, anorexia, and jaundice (Ajoy *et al*., 2010). In these cases, fatty liver and centrilobular necrosis were also noted.

The important physicochemical properties of AFB1 are odorless, tasteless and colorless. It poses a real challenge to food handlers, consumers and regulators who are in a bid to control or eradicate it since it is difficult to detect sensorically. AFB1 exists as colorless to pale yellow crystals or white powder, figure 1 shows yellow mold, caused by *Aspergillus flavus* and *Aspergillus parasiticus*, which commonly produces AFs.

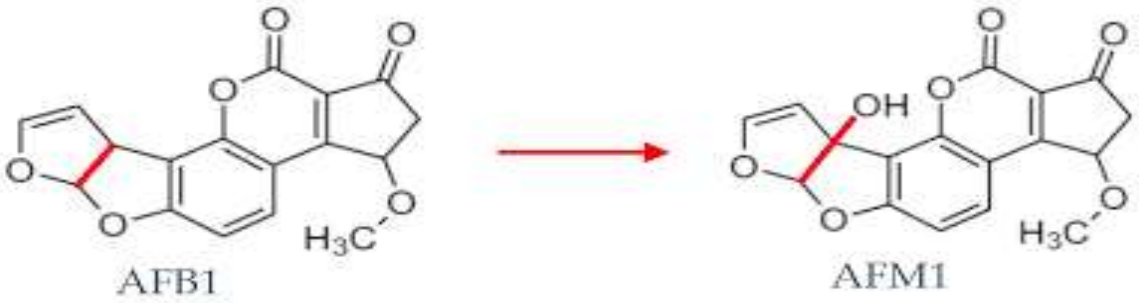
Figure 1 Yellow Mold Caused by *A. flavus and A. parasiticus*

2.6. Aflatoxin M1

Following the ingestion of AF contaminated feed by lactating dairy cows AFMI is secreted into milk. Aflatoxin M1 is a major metabolite of parent AFB1 that is formed by enzymatic hydroxylation at the 9a-position (Figure 2) and has an approximate overall conversion rate equal to 1 to 3% (Applebaum,1982). AFM1 is excreted from the body through urine, bile, feces and milk. Consequently, exposure to AFs can occur directly through contaminated food or indirectly by AFMI contaminated milk (Nishimwe *et al*., 2019).

Because AFM1 is the main hydroxylated metabolite of AFB1 carried over in the milk of lactating animals fed on contaminated feed with AFB1 (found in milk, hence the designation as M). In addition to its high toxicity and potential carcinogenicity in humans (Benkerroum, 2020a), AFM1 is of particular concern for the safety of dairy products. Aflatoxin M1 toxins share the same chemical characteristics as AFB1 toxins in that they are easily soluble in polar organic solvents, only marginally soluble in water, and insoluble in nonpolar solvents. They are resistant to heat degradation during food production thanks to their remarkable thermal stability, even at high temperatures (more than 100 °C) (Marchese *et al.*, 2018).

The range of AFB1 carryover as AFM1 in dairy cow milk has been determined to be between 0.3% and 6.2%. According to several reports on the metabolism of AFM1, cows often excrete 0.2–4% of the AFM1 they consume in the form of milk (Atanda *et al.,* 2013). As AFM1 is excreted into milk within 12 hours in the form of AFM1, with residues about equal to 1.7% of the dietary AFB1 level (Demissie, 2018), up to 6% of eaten AFB1 is introduced into milk (Nishimwe *et al.,* 2019). After 24 hours, the concentration in milk reaches its highest. Since AF stops appearing in milk four days after oral dosing ends, clearance is likewise relatively quick (Diaz *et al.,* 2004). These results confirm the rapid absorption and metabolism of AFs in ruminants. AFMI has been categorized as a class 2B (or probable) human carcinogen (Marchese *et al.,* 2018). It is of particular concern because infants and children, who are uniquely vulnerable and are potentially more sensitive to AFs than adults, are major consumers of milk.



Aflatoxin B1 **Aflatoxin M1**

Figure 2. The chemical structure of AFBI and AFM1.
Source: Marchese et al. (2018)

1.6.1. Stability of Aflatoxin M1

Several studies have been done regarding AFs and particularly AFM1 stability in milk and dairy sub products. Yousef *et al*. (1989), extensively reviewed information on the stability of AFM1. Studies have shown that there was no significant changes of AFM1 concentration after heat processing (Pasteurization or boiling) or Ultra-high temperature processing (UHT) technique (Galvano *et al.*, 1996). The stability measurements on powder milk showed no significant trends for both short- and long-term stability studies (Joseph. *et al., 2005*). In addition, studies done on AFM1 concentration changes in cheese showed no significant change of concentration even after 3 months of storage (Doveci, 2006). However, (Khoury *et al*., 2011) investigated the binding ability of AFM1 by Lactic acid bacteria (LAB) such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* and found that they were effective in reducing the extent of free AFM1 content in liquid culture medium and during yogurt processing. Therefore, this is a first study showing the capacity which can be played by LAB in AFM1 removal and could be used as a biological agent for AFM1 reduction. It is important to mention that the stability of AFM1 during processing and storage makes it dangerous.

2.7. Structure and Physical Properties of Aflatoxins

Aflatoxin was isolated and described after the Turkey X disease, which claimed the lives of over 100,000 turkey poultry after they ate contaminated peanut meal produced in South America from contaminated raw materials (Blout, 1961; Goldblatt, 1969). The AFs AFB1, AFB2, AFG1, AFG2, and the most significant AF metabolic byproducts AFM1 and AFM2 are among the 13 compounds that have so far been identified. The most hazardous variants, AFB1 and AFB2, are jointly produced by *Aspergillus flavus* and *Aspergillus parasiticus,* whereas AFG1 and AFG2 are solely produced by *Aspergillus parasiticus.*

Aflatoxin M1 and AFM2 were first discovered in the milk of cows who consumed moldy grain. Aflatoxin M1 has also been discovered in the fermentation broth of *Aspergillus parasiticus*. In an effort to make the molecules more hydrophilic so they may be quickly excreted from the body through the kidney, the animal's liver goes through a conversion process that results in these chemicals. Aflatoxin M1 is a metabolite of AFB1, and exposure at ng levels can come via mother's milk in both humans and animals. Likewise, AFM2 is a metabolite of AFB2 in milk of cattle fed on contaminated food (Santini and Ritieni, 2013).

They are chemically stable in food and resistant to degradation under standard cooking conditions, and their chemical structure includes dihydrofuran and tetrahydrofuran moieties linked to a substituted coumarin. The accumulation of AF is weather dependent, and it is difficult to eradicate once it has been created. Prior to harvest, the danger of AF development is greatest during large droughts, despite the fact that when soil moisture is low and temperatures are high, the quantity of Aspergillus spores in the air increases. These spores infect crops through insect and weather related damage (Henshall, J. D. 2012).

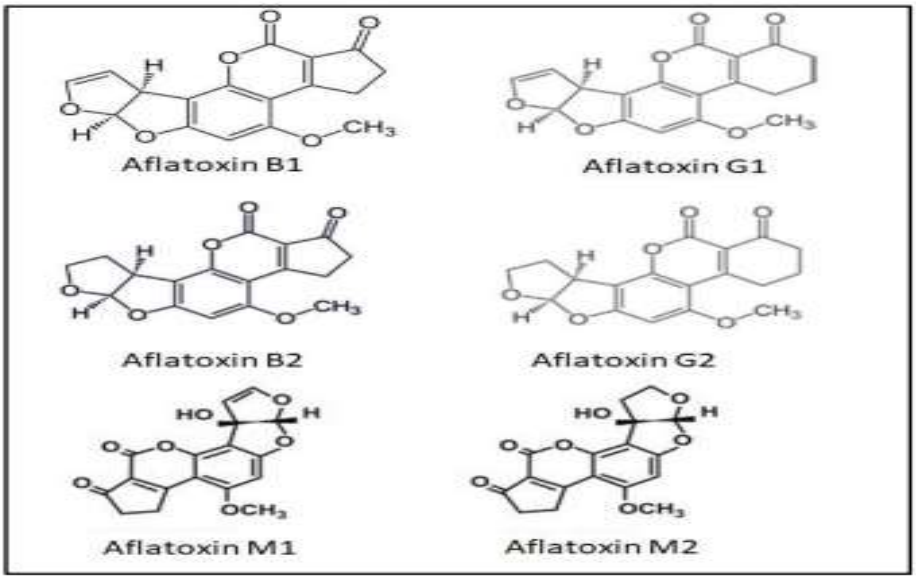


Figure 3. Chemical structures of the six Aflatoxins

Source: Henshall, J. D. 2012)

Property of AF	B1	B2	G1	G2	M1
Chemical formula	C17H12O8	C17H12O8	C17H12O7	C17H14O7	C17H12O7
Molecular weight	312	314	328	330	328
Melting point	268-2690c	287-289(D)	244-249(D)	230	299(D)
Sorbent pentane	Chloroform	Chloroform	Chloroform	Ethyl acetate	methanol
Fluorescence mission	425nm	425nm	450nm	425nm	425nm

D= decomposition

Table 1. Summary of Physical Properties and Spectral Characteristics of AFs

2.8. Health Effects of Aflatoxins in Human and Animals

Both humans and animals can develop the disease known as aflatoxicosis as a result of AFs. The liver is the only organ that AFs particularly target (Abdel-Wahhab *et al.*, 2007). The early signs of liver damage from AFs are fever, malaise, and anorexia, which are followed by abdominal discomfort, vomiting, and hepatitis; however, cases of acute poisoning are unusual and exceptional. There are two primary forms of it. The first is acute severe poisoning, which

causes immediate liver damage and ends in illness or death, and the second is long term, sub-acute exposure (Jonathan *et al*., 2004). When moderate to high quantities of AFs are eaten, acute primary aflatoxicosis results. Hemorrhage, abrupt liver damage, edema, changes in digestion, absorption, and metabolism of nutrients as well as potential death, are just a few symptoms of particular acute illness episodes (Thrasher, 2012). According to Farombi (2006), acute hepatic damage epidemics have been linked to acute dietary exposure to AFB1. Around the world, there is evidence of acute Aflatoxicosis in people, particularly in third world nations like Taiwan, Uganda, India, Kenya, and many more.

Animal nutritional status is significantly impacted by chronic AF exposure, although unlike immunological toxicities, no specific species-specific thresholds for these effects have been established. Aflatoxin quickly binds to DNA and inhibits protein synthesis following exposure, and these effects last for around 5 days (Williams *et al.*, 2004). Ingestion of low to moderate doses of AF causes chronic primary aflatoxicosis (USAID, 2012). The impacts are typically subtle and challenging to spot. Impaired food conversion and decreased growth rates, with or without the creation of overt AFs.

Exposure to AFs is primarily an issue in emerging and underdeveloped nations because of lax food storage and processing regulations and high rates of malnutrition. In the majority of sub Saharan countries, AFs have also been associated with kwashiorkor and marasmus in children. Chronic aflatoxicosis which is brought on by prolonged exposure to low to moderate amounts of AF in the food supply chain, affects a large number of people in these nations. According to (WHO, 2000), AFB1, AFB2, and AFM have been found in the liver, gallbladder, spleen, heart, muscle, and kidney syndrome are some of the prevalent symptoms .

Aflatoxicosis can affect any type of animal, and each animal's susceptibility varies greatly depending on the dose, period of exposure, species, age, sex, and diet. Animals exposed to AFs on a regular basis experience immunosuppression, as well as disruptions in the metabolism of numerous micronutrients and proteins that are essential for good health because of the development of adducts. According to Wallace (1997), these adducts are to blame for mutations, cancer, immunological suppression, lung damage, and birth abnormalities.
In animals with low levels of AFs in their feed, aflatoxicosis signs may not be visible to milk

producers. However, dairy cows may experience eye issues if there are high levels of AFs present for a long time. The main symptoms of chronic AF poisoning include an unwillingness to eat, slowed development, and impaired feed conversion efficiency. Other potential side effects include lethargy, weight loss, a rough coat, and moderate diarrhea. Abortions and irregular estrous cycles (both too short and too lengthy) are examples of how the condition might reduce reproductive effectiveness. A diminished immune response and a higher risk of contracting illnesses are other signs.

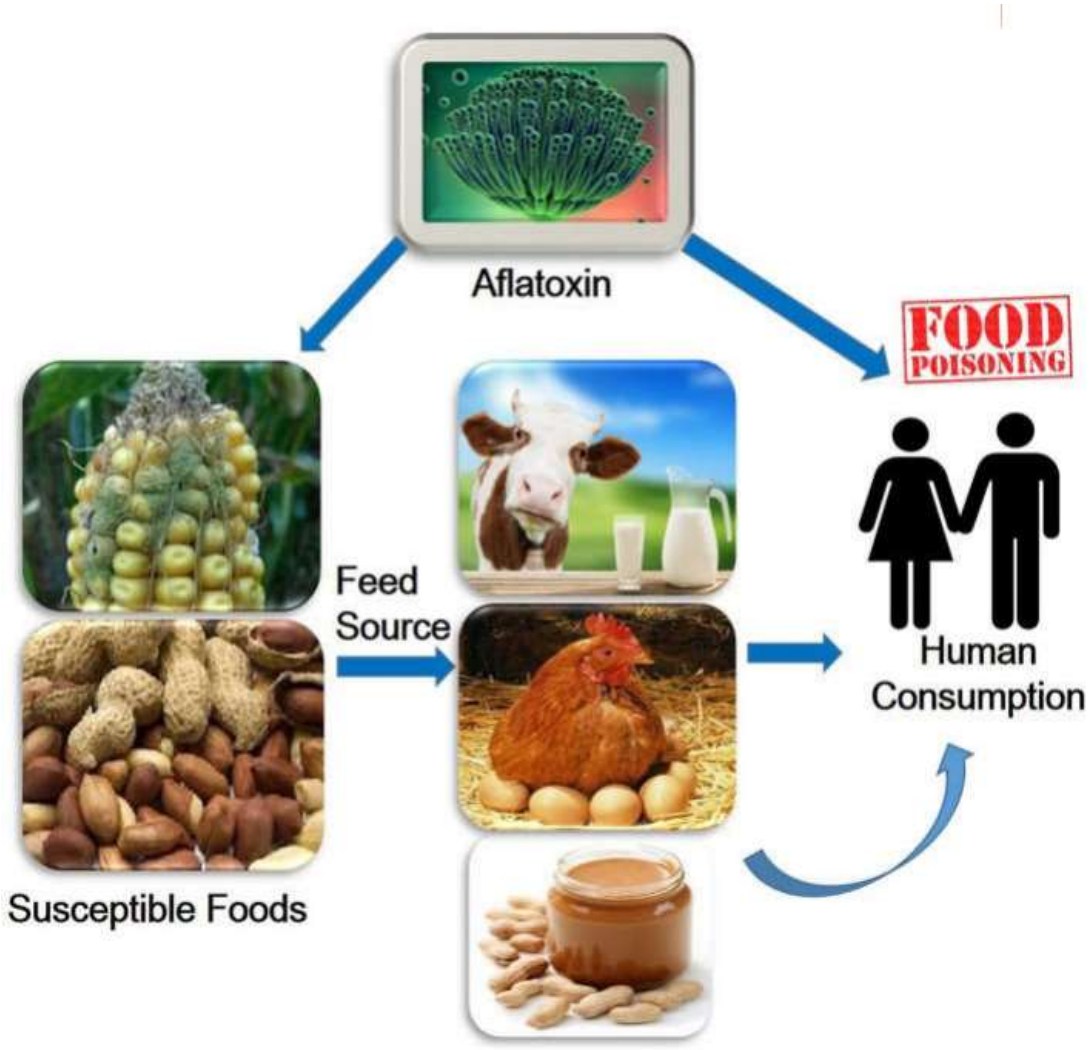


Figure 4. Overview of AF effects on humans

2.9. Economic Impact of Aflatoxins

Due to high mycotoxin levels in food and agricultural products, Africa loses an estimated $67 million in export rejects each year (Atanda *et al.,* 2013). With a significant decline in revenue over the past few years, Ethiopia has been losing market share to the European Union. Different spice items from Ethiopia that were contaminated with mycotoxins were turned away at the borders of Germany and the United Kingdom (UK) in 2015 (Firew *et al*., 2020). Similarly, from 2017 to 2019, 18 border denials on Ethiopian spice imports were made, and 10 million dollars' worth of hot pepper powder had been returned to Ethiopia from the European Union markets owing to the presence of unsafe levels of AFs (Ethiopia Capital News, 2016).

Aflatoxin M1 contamination of milk and dairy products might have negative financial effects for dairy producers. Rejecting items that don't adhere to AF standards from domestic or international markets has a direct economic impact (Balina *et al.,* 2018). An estimated potential yearly cost of $22.2 billion for dairy feed producers and an additional $37.4 million for farmers as a result of reduced milk production from cows fed AF contaminated feed (Senerwa *et al.*, 2016).

However, losses brought on by AF contamination of food can include medical and pharmaceutical expenses for the treatment of food poisoning (Demissie, 2018). Due to the low market value of contaminated goods, production rejection, and exclusion from high value markets, AFs lower farmer incomes in emerging economies (Nishimwe *et al.,* 2019). According to Atanda *et al.,* (2013), the economic impact on livestock output includes mortality in addition to declines in productivity, weight gain, feed efficiency, infertility, and disease resistance, as well as a drop in the quantity and quality of meat, milk, and eggs produced. Budgetary issues caused by food safety issues include costs associated with outbreak investigations and food recalls, national oversights resulting from increased therapeutic payments, absences from work and school due to food borne illness, consumer mistrust of the food supply, and increased demands on previously imposed and underfunded healthcare systems (Boutrif, 1998).

2.10. Tests to Detect Aflatoxins in Feed and Milk

The identification and quantification of this chemical are crucial due to its toxicity and the current maximum residue limits established. Many researchers have been interested in mycotoxin analysis that is accurate and quick. According to Rahmani (2009), various analytical techniques have been created with varying degrees of sensitivity and accuracy. It is crucial to specify the level of detail in the AF analysis because the method that can be used is constrained by the goal of the analysis: if the goal is simply to detect the analyte in the sample, rapid methods can be used; if the goal is to detect and quantify the analyte, quantitative methods must be used. Sample preparation, which may involve extraction and cleanup (purification), sample selection, and analysis (identification and quantification) are typically the processes that follow in analytical methods (Hussain, 2011).

There are several methods for the detection of AFM1 in dairy products and the choice of the method should consider the following factors: sensitivity, precision and reliability of the method, to ensure compliance with current standards. One of the most often used techniques for analyzing mycotoxins like AFs is chromatography. Gas chromatography (GC), liquid chromatography (LC), high-performance liquid chromatography (HPLC), and thin-layer chromatography (TLC) are the chromatography methods used most frequently. Of them, LC and HPLC are the most often applied. The fluorescence detection stage frequently comes after them (Cavaliere *et al.*, 2006). The most popular quantitative techniques for studying AFs are liquid chromatography (LC), thin layer chromatography (TLC), and high performance liquid chromatography (HPLC). Although these techniques have excellent sensitivity, they frequently call for expensive equipment, experienced operators, and extensive sample pretreatment (Sapsford *et al.*, 2006).

2.10.1. High performance liquid chromatography (HPLC)

Chromatography is one of the most popular methods to analyze AF which includes TLC, High Performance Thin Layer Chromatography (HP-TLC), and HPLC. TLC is also known as flatbed chromatography or planar chromatography which is used for the separation, purity

assessment and identification of AFs. In this method there is lack of precision whereas the high performance thin-layer chromatography method improves the precision by automating the sample application and plate interpretation steps. Due to their improved performance and dependability over TLC, HPLC methods are now frequently employed, and this approach has been developed for the detection of all major mycotoxins in cereals and other agricultural products. HPLC is primarily employed in the analysis of AFs for the ultimate separation and detection of the analyte of interest; extraction and clean-up procedures must be used before HPLC detection.

Chromatography columns are the most important part of the HPLC and there are two types of HPLC methods are commonly used i.e., normal and reversed phase columns are used for separation and purification of toxins depending on their polarity. In normal phase chromatography, a polar stationary phase such as silica gel and a non-polar solvent such as hexane are used whereas reversed-phase chromatography (RP-HPLC) utilizes non-polar stationary phase such as C-8 or C-18 hydrocarbons and polar mobile phase such as water, methanol or acetonitrile. AFs are naturally strongly florescent compounds, so the HPLC identification of these molecules is most often achieved by florescent detection (Goda *et al.*, 2001). Therefore, it is more preferable to analyze AFM1 from milk sample by using immune affinity column, HPLC with FD.

2.10.2 .Enzyme Linked Immuno Sorbent Assay (ELISA)

Among the screening methods, the ELISA has been the most used for AFM1 in different food matrices, such as pasteurized and ultra-high temperature (UHT) milk, infant formula, powdered milk, yoghurt, ice cream and cheese, due to its simplicity, sensitivity and adaptability (Ketney, 2017). Bioassays are techniques commonly used for screening since the immunological methods may give rise to false positives. This is because, although the antibodies are specific to their antigens, they can react with other substances (Hussain, 2011). Thus, the use of immunological methods, such as the ELISA, could be used at a preliminary stage to select from a wide range of samples those that are contaminated with the toxin under study. Subsequently, other methods are used to confirm the results. Among several methods

cited throughout the work, it has been found that those best suited for AFM1 detection in dairy products are chromatographic ones with fluorescence detection or those coupled to mass spectroscopy. Quantitative analysis of AFM1 using the ELISA method is faster and more reliable, simple and cost-effective for large sample size analysis than other methods (Matabaro et al., 2017)

2.11. Prevention Control and Methods of Aflatoxin in Milk

The first step in eliminating AF is raising public awareness of mycotoxins' occurrence, accumulation, and metabolism as well as the risks to livestock and the general public they pose due to mycotoxins contamination of agricultural crops, feed, and other food products. It is also crucial to prevent and neutralize the presence of the toxins in livestock feed and other foods derived from animals, such as milk. Additionally, feed workers need to be encouraged to regularly request safe, high quality feed and made more aware of the health concerns associated with exposure to mycotoxins in contaminated feed (Nishimwe *et al.,* 2019). Several tactics have been created more lately to help clean and/or detoxify as well as hinder the proliferation of mycotoxigenic fungus.

Aflatoxins occurring naturally in foods and feeds may be reduced by a variety of procedures. Improved farm management practices, more rapid drying and controlled storage are now defined within Good Agricultural Practice (GAP) or Hazard Analysis: Critical Control Point (HACCP) (IARC, 2002).

Aflatoxicosis or contamination of milk based products by AF can only be prevented by feeding AF free rations to dairy animal which can be enhanced through preventing fungal growth in feed and good agricultural practice in dairy production farm and processing scheme. So preventing contamination of AF needs continues plus systematic scrutiny and analysis platform (Dashti, 2009). Generally, to reduce the threat of animal and human exposure to AF requires multidisciplinary and universal methodology due to the complex nature of AF contamination ways of milk and milk products. For that reason, it requires increasing of public perception on AFs and applying scrutiny provision beside the whole dairy products. Commonly, elimination of AFs requires both direct and indirect reduction approaches of AF in

milk and dairy cow feed (Creppy and Edmond 2002).

2.11.1. Indirect Methods of AF Reduction in Livestock Feed

Using of AF polluted feed for dairy cattle typically leads to the production of contaminated milk with AFs. Hence, indirect controlling of dairy cattle feed hygiene is potential in decreasing AF contamination Strictly decreasing means of Aflatoxin contamination in the course of crop production, traditional and industrial storage of livestock feed as well as livestock feed factories are obligatory to attain the objective, value and wellbeing concerns (Dashti, 2009). Furthermore, effective ways of decreasing the contamination can be gained through proper storing of crops which embraces keeping crops on dry and clean sides, keeping crops from wetness, high temperature, pests and practice of fungicidal drugs (Wu *et al.,* 2010).

Additional central way of regulatory and decreasing AF pollution is strict observation of hygienic conditions in conventional and modern level factories and livestock feed store rooms. Monitoring growth of fungus and formation of AF in old-style farms and storages are extremely imperative (Dashti, 2009). Level of AF in milk can also be influenced by seasonal situation, for instance occurrences of AF is greater in winter and autumn compared to summer and spring due to inaccessibility of fresh feeds and farmers use warehoused forages in this unfavorable time of year. (Creppy, 2002). So, it is indispensable to advance improper storeroom and moisture conditions of livestock feed storage.

To achieve effective prevention and control of AFs, persistent and vigilant observation of diverse techniques of production and storage condition, controlling moisture content and adjustment of heat through ventilation methods, use of uncontaminated materials aided for preparing livestock feed are compulsory (Decastelli *et al.*,2005). In order to avoid mold development and inhibit growth of the molds in the early occurrences of contamination, microbes, chemicals, ionizing rays and absorbent material can be used (Shinha *et al.,* 1998). Outstanding approach of decreasing AF contamination in infected animal feed is using of AF absorbents. In the course of using absorbents, AFs existing in animal feed prevents from toxic

reactions in livestock body as well as from absorption into digestive tract by binding to absorbents (Huwig *et al.,* 2001).

In addition, prevention of AFs by chemical compounds involves series of organic acids like sorbic, propionic, acetic acids and benzoic, organic acid slats such as potassium sorbate, calcium propionate, liquid or solid copper sulfate as well as diverse microbes such as *lactobacillus brevis* and *lactobacillus pentose's* is alternative approach of decreasing AF in animals feed (Jouany, 2009).

2.11.2. Biological methods

Advances in the field of biotechnology have opened a new avenue for tackling mycotoxicosis. A natural organic product, glucomannan containing yeast product, a cell wall derivative of *Saccharomyces cerevisiae* have shown considerable binding ability with commonly occurring mycotoxins (Devegowda and Murthy 2005) and is found beneficial in minimizing the adverse effects of mycotoxins in livestock and poultry. Using plant extracts to reduce AFM1 in milk. One interesting technic to diminish toxins, especially AFM1 in foods, is using the plant extracts. Some plants such as broccoli, garlic, black cumin, and curcumin have been reported to be efficient for reduction of AFs. The mentioned extracts may also have inhibitory effect on AFs. The extracts are also known as antioxidant, antifungal, and anti-inflammatory agents because their ability to inhibit infection or inflammation inducing molecules (Raju and Devegowda 2002).

2.11.3. Broccoli, garlic, and black cumin for reducing AFM1

Broccoli extract is an attractive novel additive with indirect antioxidant properties. In a study, it was shown that the broccoli extract has the ability to reduce the AFM1 content in milk. This is important since milk is consumed by humans especially children (Akram *et al.*, 2010). Water extracts of garlic, black cumin, and carrot were attempted to use for detoxification of AFM1. The level of AFM1 was significantly reduced by adding the mentioned extracts to milk in ratio of 1:10. There are limited data about ability of such extracts to reduce the toxin in milk, but above mentioned finding may reflect the advantages of plant extracts to detoxify AFs in dairy products during fermentation stages. (Ayoub *et al.*, 2011*)*

2.12. Limits of AFs in Feed and Milk

Strictly speaking, there is no safe level with reference to mycotoxins, the risk directly depends on the level of the major mycotoxins and also on the presence and levels of other mycotoxins in feeds. A mycotoxin level considered safe in one farm may not be safe in another farm because of differences in management and disease prevalence. Additionally, factors such as interaction of mycotoxins with pathogens, genetic variability, environmental conditions, and nutritional status etc. influence the severity of the manifestation of mycotoxicosis. (Atanda *et al.*, 2013).

In order to reduce the toxic and economic impact of mycotoxins, several countries regulate the levels of some mycotoxins in foods and feeds. Worldwide, food and feed legislation safeguards the health of consumers and the economic interests of animal producers and traders. Virtually, all countries with fully developed market economies have regulations with the exception of some African countries. These regulations may include specific maximum limits for several contaminants for different foods and feed. Consequently limiting AFB1 in feeds is the most effective means of controlling AFM1 in milk (FAO, 2011).

Aflatoxin regulation has great effect on international trades, especially for the developing countries like Ethiopia. In these countries, where food supplies are already limited, legal measures may lead to lack of food and to excessive prices (Wolde, 2017). The Ethiopian Standard Agency (ESA) has established the maximum limits of AFs in feed components and compound feeds for various animal categories by the year 2019 (Table 2) and has determined that the maximum AF limit is required owing to its effects on the community's health. A diagram showing how AFB1 becomes AFM1.

More than 80 countries monitor AFs, but there is no worldwide harmonization of their legal systems. As a result, according to Iqbal *et al*. (2015), the permissible limit for AFM1 in milk and dairy products is between 0 and 1.0µg/kg. Aflatoxin M1 levels in raw milk, heat treated milk, and milk used to make dairy products in the EU should not be more than 0.05µg/kg for adult consumption and 0.025µg/kg for baby and young child food products. On the other hand,

a limit of 0.5µg/kg for milk was established by the FDA (United States) and the Codex Alimentarius Commission. The strictest laws governing mycotoxins in food are found in the EU (Jaiswal *et al.*, 2018).

Due to AFM1 health hazard potential, it is therefore important to monitor and regulate the level of AFM1 in milk and milk products through control of feed quality for consumer's safety purposes. In Ethiopia, Ethiopian veterinary drug and feed regulation and Control System has been enacted by Proclamation No. 728/2004 and Council of Minister Regulation No. 299/2013, with main responsibility given to veterinary drug and animal feed Administration and Control Authority (VDFACA), and some of the responsibility shared among sector ministries such as Ministries of Agriculture, Trade, Industry. In 2015, VDFACA implemented Animal Feed Registration Directive number 02/2010, whereby issues related to feed safety and contaminations as well as appropriate measures based on the legislation have been clearly indicated.

Feed type	TAFs ppb	AFB max.
Compound feed for pregnant and lactating dairy cows; dairy heifer *Noug* cake (meal) as feed ingredient	40	20
Compound feed for fattening cattle, sheep, goat and ewes, breeding rams, does, bucks; Linseed, Rapeseed, Sunflower and Sesame cake (meal) and Wheat milling by-products as feed ingredient	100	50
Compound feed for tilapia and catfish	20	10
Cotton Seed and Groundnut cake (meal), Maize and maize processing byproducts as feed ingredient	200	100
Brewery by-products as feed ingredient	50	20

Table 2. Ethiopian standards of AFs in feed/feed ingredients

Source: ESA (2019a-w)

Country	AFB1
United States of America	20ppb
European Union (for milking cows)	5ppb
European Union(for calves)	10ppb
India	20pp

Table 3. Limits for AFB1 in dairy feed

Country	AFM1
Ethiopia	0.5µg/l
United States of America	0.5µg/l
European Union	0.05µg/l
European Union for baby foods/infants	0.025µg/l
Australia	0.05µg/l
Australia ,for infants	0.02µg/l
India	0.5µg/l

Table 4. Limits for AFM1 in milk

2.13. Status of Aflatoxin in Ethiopia.

Due to predisposing pre-and post-harvest factors like frequent and seasonal drought (soil and water stress), a lack of resistant varieties, harvesting techniques, storage facilities, and conditions (sanitary level, pest, moisture level), low or limited knowledge of AF by value chain actors, a lack of a regulatory framework, and a lack of monitoring facilities, the status of AF in Ethiopia is widespread, according to numerous studies conducted in the country. (Alemayehu, 2014)

Ethiopia is the most conducive country for AF pollution, particularly AFB1, which results in AFM1. Numerous research revealed that the most prevalent AF in the nation, AFB1, and the greatest AF, AFM1, which is shown to be more prevalent than the norm in milk produced, have a significant impact on animal feeds. This suggests that AF contamination in milk is a

concern for many developing nations, including Ethiopia. Health issues for humans and animals are more likely when AF exposure is not known about. The nation's livestock feed and milk production suffer significant economic losses as a result of AFs. Human health may be at risk from AFB1 in animal feed and AFM1 in animal milk. High levels of AF contamination in animal feed (AFB1) may result in a significant AFM1 level in milk when animals are fed with highly contaminated foodstuffs. Based on the AF content (26 mg/kg) in common grains AF exposure in Ethiopia was predicted to be between 1.4 to 36ng/kg body weight/day. In addition, Ethiopia has one of the highest hepatocellular carcinoma mortality rates in the world (93.4 per 100,000) (Tirmenstein and Mangipudy, 2014). Chronic low level exposure to AFs may also lead to impaired immunological function, malnutrition, and stunted growth in children (Benkerroum, 2020b). The presence of AFs (B2, G2, and M1) in 17% of the urine samples from children in the Amhara and Tigray areas of Ethiopia revealed that AF exposure is a serious issue for children's health in Ethiopia. In addition, Ethiopia has a higher rate of childhood malnutrition, with 38% of children (under 5 years old) being stunted.

For instance, cross-sectional study was conducted in by Zebib *et al.* (2022), in three regions of Ethiopia, Oromia, Southern Nations, Nationalities and People (SNNP), and Amhara regions on AFM1 from producers, collectors, processors and retailers. A total of 160 composite samples were collected and analyzed. AFM1 was detected in all milk products with contamination percentages of 62.50%, 67.20%, 25% raw milk, pasteurized milk and cottage cheese respectively and they were above the regulatory limit set by the EU (0.05 mg/L).

Mesfin *et al.,* in (2018) in the central highlands of Ethiopia particularly in Holetta, Bishoftu and Hawassa indicated contamination of milk in the range of 0-0.146; with a mean AFM1 of 0.054µg/l. A cross-sectional study was conducted by Admasu *et al.* (2021), in South Gondar Zone to evaluate the concentration of AFM1 and was detected in 99 analyzed milk samples. Another study conducted by Dawit *et al.* in (2016), in greater Addis Ababa milk shed. There was high level of AFM1 contamination. From Dairy farms all 100 milk samples were contaminated with AFM1. Highest was 4977 ppb and lowest was 28 ppb. 92% exceed FAO/WHO and EU limit of 0.50µg/l and 0.05mg/l respectively. Overall only nine (8.2%) out of a total of 110 milk samples contained less than or equal to 0.05µg/L of AFM1.

Furthermore, 29 (26.3%) milk samples exceeded 0.5µg/L.

2.14. Global scenario of Aflatoxins

Aflatoxins, the most widespread of all the mycotoxins, are common in warm and humid climatic conditions like those existing in India, Latin America, Asian, and African countries, the southern regions of the USA, and certain parts of Australia. Extensive surveys conducted in India, Pakistan, Egypt, and South Africa suggested that AFs are often encountered in substantial levels in feeds and feed ingredients. In Latin American countries including Brazil, Peru, Mexico, Columbia, Venezuela, and Argentina, reports exist on the presence of AFs. Due to the increase in global trading of feedstuffs, mycotoxins are no longer solely found in certain geographical regions but are now more widely distributed than before (Devegowda *et al.*, 1998). Among all, AFB1 is the most prevalent and toxic.

Based on the AF content (26 g/kg) in common grains, AF exposure in Ethiopia was calculated to be between 1.4 to 36ng/kg body weight/day. In addition, Ethiopia has one of the highest hepatocellular carcinoma mortality rates in the world (93.4 per 100,000) (Tirmenstein and Mangipudy, 2014). Chronic low-level exposure to AFs may also lead to impaired immunological function, malnutrition, and stunted growth in children (Benkerroum, 2020b). The presence of AFs (B2, G2, and M1) in 17% of the urine samples from children in the Amhara and Tigray areas of Ethiopia revealed, according to Firew *et al.* (2020), that AF exposure is a serious issue for children's health in Ethiopia. Ethiopia also has a higher rate of childhood malnutrition, with 38% of children (under the age of 5) suffering from it.

3. MATERIAL AND METHODS

3.1. Description of the Study Area

This study was conducted between December 2022 and April 2023 in dairy farms and collection centers found in and around Adama town, East Shewa zone central Ethiopia. East Shewa Zone has a total of 1,147,173 cattle (CSA, 2021). Adama is one of the districts in the East Shewa Zone, central Ethiopia, at about a distance of 99 km south-east of Addis Ababa (39.17°E and 8.33°N), with an altitude of 1622 meters above sea level in the rift valley. The town is situated along the road that connects Addis Ababa to Harar and Dire Dawa. The area receives an average annual rainfall ranging from about 600 to 1150 mm, which is erratic in nature. There is a significant seasonal variation in the amount of rainfall. More than 67% of the mean annual rainfall occurs in the four rainy months: June, July, August, and September. Some additional rains (about 23%) occur in the remaining dry months, with mean monthly values of rainfall as low as zero millimeters. The minimum and maximum daily temperatures of the area are 12℃ and 33℃, respectively (CSA, 2021).

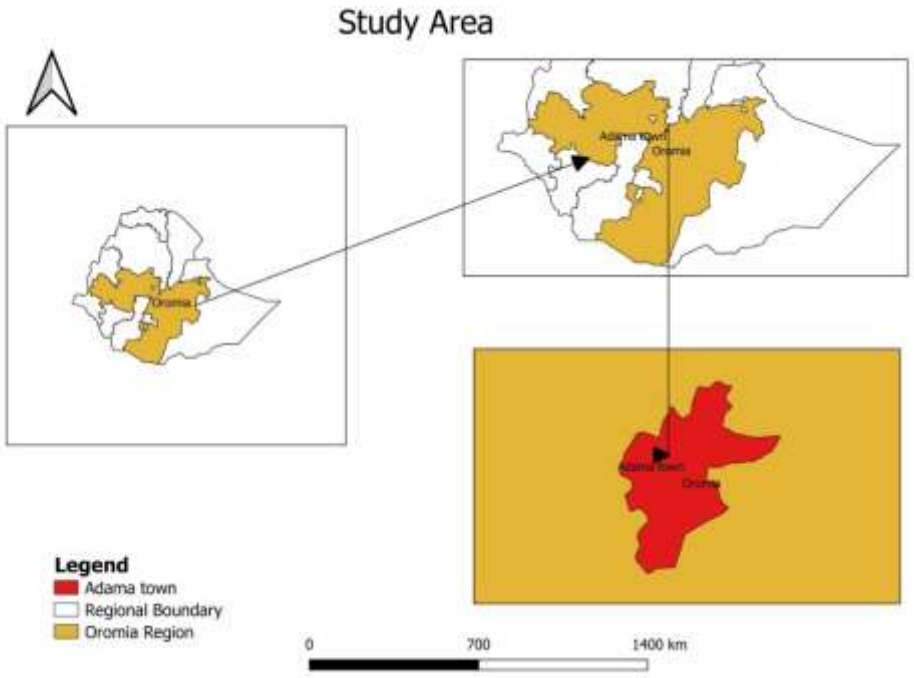


Figure 5. Map of study area

Adama district was purposively selected based on dairy farm availability. Urban and peri

urban production systems were included. According to Adama special zone agricultural office data, there are around 800 small scales, 200 medium and 48 large scale dairy farms are found. Different feed mill produce feeds for sale, different factories, feed suppliers and feed traders are located. Hence this area has a greater potential of dairy production and accessible for dairy and beef cattle feed and feed ingredient.

3.2. Study Design

A cross-sectional study design was conducted on 120 raw cow milk samples collected from December 2022 to April, 2023 with the aim of detecting and quantifying the level of AFM1. The milk samples were collected from dairy farms and milk collection centers in and around Adama town, central Ethiopia. Adama town was purposively selected based on dairy farm availability. Urban and peri urban production systems were included. According to Adama zone agricultural office data, there are around 800 small scales, (3-6), 200 medium (7-15) and 48 large scale (>16) dairy farms. Different feed mill produce feeds for sale, different factories, feed suppliers and feed traders are located. Hence this area has a greater potential of dairy production and accessible for dairy and beef cattle feed and feed ingredient. A simple random sampling technique was applied to get dairy farms and collection centers. The study was conducted on a total of 120 raw cow milk samples of which 95 were collected from dairy farms and 25 were obtained from milk collection centers.

3.3. Sample Size Determination

The approximate sample size was determined according to Thrusfield (2005), formula as shown below. Considering previous AFM1 prevalence of 91.8 % in milk by (Dawit *et al*, 2016) in greater Addis Ababa milk shed. Desired precision of 5% and confidence interval of 95%. Based on this prevalence, a totally 120 raw milk samples were collected from dairy farms and collection centers.

$$n = Z^2 P_{exp}(1 - P_{exp}) \quad n = \frac{(1.96)^2 (0.918(1-0.918))}{0.005} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$
$$d^2$$

where: Z= confidence level of 95 %

n = required sample size;

p exp = expected prevalence;

d = desired absolute precision

n=115

3.4. Milk Samples Collection

100 ml of pooled raw cow milk was collected using 50 ml sterile falcon tubes from dairy farms and milk collection centers (APITC, 2022; AOAC 2002). The milk was mixed and homogenized properly in collecting tank of each farm, and representative sample was taken directly from the tank. After the samples were taken, all the necessary information like the location of the samples, the date of sampling, the name of the person who took the samples, and the batch or code number, were registered on the samples. The samples refrigerated and transported using coolers (an icebox) at a temperature between 2 and 5 °C to the Animal Products and Inputs Quality testing Center (APIC) of the Ethiopian Agricultural Authority, where they stored at −20 °C until the respective analysis.

Along with sample collection, a set of questionnaire survey was conducted on all (95) dairy farmers from which milk samples were collected. Data was also collected with reference to the general characteristics of the dairy farms, like the number of animals and farm management practices, which would be analyzed as potential risk factors (source of feed, storage place and time, moisture control) and attitudes towards AFs associated with the presence of AFM1 in milk and attitudes towards AFs. Physical observation was conducted to determine the general condition of the farms (ventilation, humidity, and temperature). To make communication easy, all the interview questions were translated into Amharic or Afan Oromo and conducted in either of the two languages as per the respondents' interests. The interview was conducted in the form of friendly dialogue to enable the participant to feel free, relaxed, and confident. The checklists were filled out by the investigator, and the actual practices of dairy farm owners at individual farms were observed.

3.5. Materials and chemicals used

3.5.1. Consumables and other materials used.

The following analytical grade chemicals, reagents, deionized water and other consumables were used in the research. Immuno affinity column, AFM1 standard solutions, and AFM1 standard stock and working solutions, HPLC grade Acetonitrile, and Methanol, and deionized or HPLC grade water.

3.5.2. Equipment

Major equipment and glassware used in the research were: HPLC system with Diode Array Detector coupled with FLD/ Fluorescence detector, Reversed phase HPLC analytical column (3µm or 5µm octadecyl silica packing and a guard column), vacuum manifold, refrigerated centrifuge and Analytical balance, nitrogen gas streamed automated sample concentrator, syringe barrels (20 and 50ml capacity), sonicator, vacuum system (to suck samples via immunoassay columns), water baths, Filter paper (Whatman No.4 or equivalent) disposable syringes (10ml and 50ml) and syringe filters. The basics glassware includes: measuring cylinder (100ml, 50ml), beakers, of capacity 250ml, graduated conical glass tubes (with ground glass neck and stopper of capacities 5ml, 10ml and 15ml), Pipettes (1ml, 2ml, 5ml, 10ml and 50ml), volumetric flask (100ml),

3.6. Sample preparation procedures

The official AOAC (2002) technique was used to guide the extraction and cleanup steps for sample preparation. In order to remove fat and other impurities, centrifugation and filtration represent key stages in the extraction process. The purpose of extraction is to remove mycotoxin from the matrix, in a suitable solvent. 50ml milk sample was taken from deep freeze and defrosted using a water bath of 40°C for 30 minutes to bring it to room temperature. Then Centrifuge it at 4500 revolutions per minute for 15 minutes to remove fat and other impurities. The fat is simply separated from the milk with a spoon. Then it was filtered with syringe filters through Whatman No. 4 filter paper to remove other impurities and transfer it into a 50ml tube. The main objectives of the clean-up step are the elimination of the matrix

interferences and analyte pre concentration (ketney *et al*., 2017). Usually, clean-up is applied after extraction to get more accurate and precise results. Commonly used purification methods employ IAC or one-step multifunctional clean- up columns. Immunoaffinity columns are a very efficient technique of purification: they are based on the recognition of the toxin by a specific antibody. Although IACs are easy to use and have high selectivity, they are single use because of the denaturation of antibodies during the elution step, and as such the costs are high.

3.7. Laboratory Analysis

Then 50 ml of defatted (skim) milk was completely passed through the Afla M1TM immune affinity column (Afla CLEAN, produced by LCTech GmbH, Germany) at a rate of about 1-2 drops per second. Then the column was washed with 10 ml of distilled water at a rate of 1-2 drops per second. Using a nitrogen concentrator, residual water was removed by a gentle nitrogen gas stream. Then 3 ml of acetonitrile was added, and we waited for 5 minutes to break the analyte-antibody bond. After 5 minutes, the columns were opened, transferred into a 10 ml centrifuge tube, and evaporated under a nitrogen stream. Finally, the samples were reconstituted with 1 ml of the mobile phase solution of water-acetonitrile-methanol (60:25:15) and transferred to an amber glass vial ready for HPLC detection (AOAC, 2002; APITC, 2022).

3.7.1. HPLC conditioning and injection procedures

The HPLC machine was conditioned by pumping a mobile phase solution of water-acetonitrile-methanol (60:25:15) at 1 ml/min flow rate until a stable baseline developed. Working standard solutions were prepared at concentrations of 0.05, 0.1, 0.5, 1, 1.25, 1.5, 2, and 4µg/l AFM1 in the mobile phase to construct the calibration curve (Fig 6). The optimal conditions of the instrument were checked with an AFM1 calibration solution before analyzing the test sample. Then, the linearity of the injection of AFM1 standard solutions and stability of the chromatographic system were checked, and 10µl AFM1 solution was repeatedly injected until a stable peak area was obtained. Peak areas corresponding to consecutive injections were within 5%. After the HPLC output, the calibration graph was

prepared by plotting the peak area against the mass of the injected AFM1. By using the same conditions as for the standard solutions, the test samples were injected by creating a batch in an ordered sequence (AOAC, 2002).

Once, AFM1 peak area is determined, AFM1 concentration in test sample was calculated from the calibration graph in μg/l. The formula used to calculate:

$$AFM1 = Wa \times (Vf/Vi) \times (1/Vs) \quad \text{..... (2)}$$

Where, W a= HPLC reading (μg/l) x Volume injected elute in to HPLC (μl)/1000. VF = The final volume of dissolved elute (ul). Vi= Volume of injected elute in to HPLC (μl). Vs= the volume of prepared test portion passing through the column (ml).

The HPLC system was interfaced, via network chromatographic software (Agilent Chem Station), to a personal computer for instrumentation control, data acquisition and processing. The result was interpreted according to Compulsory Ethiopian Standards (CES 278) a regulatory limit set by Ethiopian Standard Agency (ESA), which is 0.5ug/l (PPb) in raw/liquid milk (CES, 2021).

3.7.2. Data management and analysis

All collected questionnaire survey data and the AF test result reports were collected from APIC, and the level of AF was categorized into comply (legal, safe) and not comply (not safe) based on the Ethiopian feed and milk standards, which are adopted from CODEX standard, which may help to characterize the distribution of AF in the study area. Samples were considered not compliant if the AFM1 level of the sample exceeded the maximum residue limit (MRL) of the standard or comply if it was below the MRL. All collected data (laboratory test result and KAP survey) were organized, coded and entered into Microsoft excel spread sheet 2010. Descriptive statistics (Maximum, minimum, mean, and Standard deviation) was used to present the result of AFM1 contamination level of milk. Table of frequency and percentage were used to figure out the finding of the questionnaire survey.

To determine the association of different risk factors with the presence of AFM1 in milk samples (positive or negative), the analysis was performed using STATA software in two steps. In the first step, all the selected explanatory variables were compared with the dependent variable (univariate analysis) using the Chi-square. AFM1 occurrence (positive or negative) obtained in dairy farms milk was used as a dependent variable, and other qualitative factors were used as explanatory variables. In the second step, linear logistic regression was used to study the association between AFM1 contamination level and the considered risk factors individually.

3.7.3. Data quality assurance

Sample collection, handling, storage, and extraction were made based on scientific protocols, and all milk sample analysis was done based on scientific standard laboratory procedures (proper hand washing, wearing latex gloves, and laboratory coats). Proper sterilization and disinfection techniques for instruments were developed based on international standard procedures.

4. RESULTS

4.1. Household Characteristics

There were All the 95 dairy farmers took part in the interview gave 100% response rate. The number of dairy cattle possessed by the milk producers that were surveyed ranged from 1 to 140 animals, with an average of more than 9animals per individual farm. Since we scheduled our sampling time to coincide with the two periods per day that the animals are milked, early in the morning or late in the afternoon, almost all of the animals were being milked at the time of the interview. Holstein-Friesians, Jerseys, and Ayrshire were among the exotic cattle breeds that all 95 of the dairy farmers raised. Sheep, chickens, and donkeys were among the additional livestock breeds preserved.

4.2. Respondents Characteristics

The demographic data showed that, males made up 72.6% of the farm owners surveyed, females 27.4%, and their ages ranged from 18 to 30 (18.9%), 31 to 45 (57.9%), and over 45 (23.2%). The respondents' educational levels ranged from complete illiteracy (26.3%) to the highest level, having obtained a higher education degree (6.3%). Only 3.2% of the respondents were farm attendants or employees, leaving almost all of them (96.8%) to be farm owners.

On feeding practice, they have reported that they supplemented their animals with commercial concentrates. The management systems of the animals varied across households with majority of households practicing zero gazing (84.2%) and least number of households practicing pasture grazing (15.8%). Farmers who practiced pasture-grazing also supplemented their animals with either commercial or compounded feed. The most commonly purchased feed was concentrates while cut and carry was obtained mainly from their own farms. The different feed types used in the households were stored either on the floor or on a raised surface of which hay, cut-carry pasture and concentrates were reported to be stored on a raised surface like pallets

Statement	Frequency	Response	Percentage
Age	18-3	18	18.9%
	31-45	55	57.9%
	more than 45	22	23.2%
Gender	male	69	72.6%
	female	26	27.4%
Education	illiterate	25	26,3%
	primary	51	53.68%
	secondary	13	13.7%
	tertiary	6	6.3%

Table 5. Respondent Characteristics

4.3. Aflatoxin M1 level of the overall Raw Cow Milk Samples

By using HPLC, the prevalence of AFM1 was detected and quantitatively measured, and the findings of the analysis were compared to national and international regulatory standards or limits, of the Codex Alimentarius Commission (CAC)/FDA/ESA, and EU. In order to calculate the prevalence, the HPLC findings of milk samples that were examined and exceeded FDA/ESA (0.5 µg/l) are employed. The HPLC analysis result showed that milk samples had different levels of AFM1 contamination. The prevalence of AFM1 was 60.8% that means 73 (60.8 %) of milk samples were contaminated with AFM1 at various degrees. However, only 11 (9.2%) from the overall 120 examined raw milk samples were found to have AFM1 residues above the CES 278 level (0.5 µg/l) and unfit for human consumption where as 42.5% exceeded the EU's acceptable limit of 0.05µg/l. The measured concentrations of AFM1 were ranging from 0.05µg to 8µg/l. The standard deviation is 0.9245µg/l, while the mean is 0.253µg/l as depicted on Table 6 below.

Location	No of animals	Positive (%)	Above 0.5 µg/l	Above 0.05 µg/l	Min µg/l	Max µg/l	Mean µg/l	STD µg/l	Unfit * (%)
Adama	120	73 (60.8%)	11 (9.2%)	51 (42.5%)	0.05	8	0.25	0.92	11 (9.2)

***** Unfit for human consumption as per CES 278

Table 6. Aflatoxin M1 prevalence and compliance status in the study area

4.4. Aflatoxin M1 level in Milk collected from of Dairy Farms

A total of 95 raw milk samples from dairy farms were collected and analyzed. Fifty one percent of milk samples were contaminated with AFM1 with different degrees. From these 6.3% and 34.7% milk samples were above the permissible limit of CES/ ESA and EU respectively. The range of AFM1 was from 0.05µg/l -0.655µg/l. The minimum and maximum AFM1 level in milk samples taken from dairy farms was 0.05µg/l and 0.655µg/l respectively. The mean and standard deviation were 0.0965µg/l and 0.1646µg/l as summarized below in table 7.

4.5. Aflatoxin M1 in Milk collected from Collection Centers

Totally 25 raw milk samples were analyzed in this investigation, and the findings indicated that 96% of the samples were contaminated with AFM1. The range is from 0.05-8µg/l. From the total of 25 milk samples 20% and 72% of these samples are above the CES/ES and EU permitted limit respectively. The mean was 0.8506µg/l and the standard deviation is 1.9139µg/l as indicated in table 7 below

Location (System)	No of animals	Positive (%)	Above 0.5 µg/l	Above 0.05µg/l	Min µg/l	max µg/l	Mean µg/l	STD µg/l
Adama (Dairy Farms)	95	49 (51.6)	6 (6.)	33 (34.7)	0.05	0.65	0.096	0.164
Adama Collection Center	25	24 (96)	5 (20)	18 (72)	0.05	8	0.85	1.91

Table 7. AFM1 Level in raw milk samples from dairy farms and collection centers

4.6. Comparing AFM1 level of Milk collected from Dairy Farms and Collection Centers

In this study, the prevalence of AFM1 in dairy farms (51.6%) is lower than the prevalence of milk samples collected from collection centers (96%). From positive ones 6.3% farms and 20% of collection centers were above CES/ES acceptable limit whereas 34.7% of farms and 72% of collection centers are above the permissible limits of the EU. The maximum concentration of AFM1 detected in dairy farms was 0.655ug/l and 8ug/l in milk collection centers, as indicated in Table 7 above.

4.7. Assessment of Dairy Farm Owners' Knowledge Attitude and Practice

Questionnaire Survey information was gathered from dairy farm owners while milk samples were being collected to know their Knowledge, Attitude and Practice regarding mold growth and conducive conditions for the formation of toxin. Data on dairy farm owners feed management practice, which are risk factors for the development of AFs, was also gathered. In order to determine whether there is a statistically significant correlation between these risk factors and the development of AFM1, we attempted to link the response of dairy farm owners with the outcome of the laboratory test for AFM1. The owners of the dairy farms were also indirectly observed as they managed their farms. Table 8 provides a summary of the outcome.

S.N	Attitude on mold growth and toxin formation	Response	Frequency	Percentage
1	Concept on mold and formation of toxin	Yes	72	75.8%
		No	23	24.2%
2	Knowledge on favorable conditions for mold growth	Yes	73	76.8%
		No	22	23.2%
3	Do you know or heard of AF	Yes	72	75.8%
		No	23	24.2%
4	Are you aware that AF cause disease in animals and humans?	Yes	40	42.11%
		No	55	57.89%
5	Does AF killed by milk pasteurization?	Yes	73	76.9%
		No	22	22.11%

Knowledge on Farm management practice				
6	Storage place of dairy cattle feed	In house	44	46.3%
		In shade	30	31.6%
		Open field	21	22.1%
7	Do you regulate moisture of the feed?	Yes	46	48.42%
		No	49	51.58%
8	Feed storage time	One week	33	34.7%
		Above 1 week	62	65.3%
9	Feed source	Grazing	15	15.8%
		Non grazing	80	84.2%
10	Do you check quality of feed while buying or feeding?	Yes	63	66.32%
		No	32	33.68%

Table 8. Assessment of KAP of dairy farm owners on mold growth and toxin formation

Of the 95 dairy farmers who were all questioned for KAP about mold growth and favorable conditions, they have a thorough extensive knowledge of it. 75.78% of respondents are very knowledgeable about how mold develops and how toxins are made, whereas just 24.22% are not. In a similar way, where as 23.15 percent are unaware of favorable conditions for mold growth, 76.8% are aware that heat and moisture are good conditions for the growth of mold (shagata). Only 24.22 percent of them do not realize that AF is present in feed, compared to 75.78% of those who are aware of it. On the other hand, just 16.84% of respondents said they understood what AF in milk was, and 83.16% said they were unaware that AF can contaminate milk.

During our questionnaire survey 42.11% of the respondents claimed they are aware that AF causes sickness in both humans and animals, as opposed to 56.84% of respondents who did not realize that AF may do so. The majority of individuals feel that pasteurization will destroy AF because they anticipate that boiling milk will remove the majority of milk contaminants. Traditional milk therapies do not, however, kill AF since it is a poison rather than a living

organism. However, just 23.11% are aware that pasteurization cannot destroy AF.

With regard to their farm management practices, 46.3% of respondents store their feed in house and routinely monitor the temperature, ventilation, moisture, mold growth, and dryness by using pallets, crates and by putting the feed on raised materials far from the walls and roofs and by protecting pest access into the store. But 51.58% of them have no ventilation system for their storage houses. 31.6% and 22.1% of them keep their feed in shade and on an open field, respectively.

The percentage of respondents who physically inspect their feed while purchasing and feeding it to their animals is 66.32%, whereas the percentage of respondents who do not do so is 33.68%. The majority of dairy farm operators feed concentrate to their lactating cows. The vast majority of them (84.1%) engage in no grazing. Their dairy animals are closely managed and supplemented with commercial concentrate, pasture, and fodder because there isn't enough grazing land in the area, which increases the risk of AF contamination. The storage time of dairy cattle feed has a significant effect on feed contamination by AFM1, but most farm owners 65.3% store their feed for more than one week. Because of market fluctuations and shortages of feed in the market, they are forced to buy bulk feed once and store it for a long time. But the rest 34.7% store the dairy feed for one week and below. Most interviewed dairy farm owners 88.4% discard animal feed if they notice fungus on it. 11.6% of them use mold-infested feed for their animals, and none of them sell this contaminated feed to others.

4.8. Association of Risk Factors with Contamination of Milk with Aflatoxin M1

Only 15.8% of the 95 dairy farm owners whose sources of feed for their dairy cattle were investigated employ a mixed grazing method, which indicates that although the cattle are housed intensively, they occasionally leave the farm and graze in the field. However, the majorities of them, 84.2%, keep their cattle intensively and give them concentrate from a commercial source. Accordingly, Table 11 below shows that 6.6% and 61.25% of milk from grazing and none grazing feed sources are positive for AFM1 contamination during laboratory analysis of milk. In the course of my observation and interview, i discovered that 34.7% and 65.3% of them held dairy cattle feed for one week and more than one week, and 30.3% and

65.3% of milk had been infected with AM1 respectively. Six of those who keep feed for longer than a week exceed the FDA regulatory limit, while 29 exceed the EU regulatory limit. 46.3%, 31.6%, and 22.1% of dairy farm owners store their feed in buildings, shaded areas, and open fields, respectively. The level of AFM1 contamination in milk from individuals who store it in an open field is the greatest of all, at 80.9%, followed by that from those who store it in a shaded area (53.3%) and inside a building (36.3%). Unless it is effectively controlled on the farm, moisture is one of the biggest risk factors for the formation of AFs in feed. According to their response, 52.6% of dairy farmers use ventilation, pallets, and crates and kept the feed on raised surfaces away from walls and roofs to reduce moisture in feed storage facilities. Laboratory testing on samples from these farms revealed that 32% of them are contaminated with AFM1, which is less than the 71.1% contamination rate of AFM1 for facilities that did not control moisture.

Variables	**No of samples**	**Positive**	**Above 0.5 µg/l**	**Above 0.05µg/l**	**P value**
Dairy farms	95	49(51.6%)	11	33	
collection centers	25	24(96%)	18	72	0.00
Grazing feed source	15	1(6.6%)	0	1	
Non grazing feed	80	48 (60%)	5	32	0.010
1week storage	33	10 (33.3%)	0	0	
Above 1 week storage	62	39 (62.9%)	6	29	0.010
In house	44	16 (6.3%)	3	13	
In shade	30	16 (53.3%)	2	18	0.009
Open air	21	17 (80.9%)	1	2	
moisture control	50	16 (32%)	1	13	0.003
no moisture control	45	32 (71.1%)	5	20	

Table 9. Association of Contamination level of AFM1 with considered risk factors

5. DISCUSSION

The current results were better compared to the study conducted by Zebib, *et al.* (2022), to assess the level of AFM1 from raw milk, pasteurized milk and cottage cheese in Oromia, Southern Nations, Nationalities and People (SNNP) and Amhara regions from producers, collectors, processors and retailers. All milk products were contaminated with AFM1 in all study regions. From raw milk samples 21.8% and 62.50% were above the regulatory limit set by FDA/ESA (0.5µg/l) and EU (0.05µg/l) respectively. From all animal products 56.88% and 19.38% were above the EU and FDA/ESA regulatory limit. Dawit *et al.* (2016), in great Addis Ababa milk shed areas reported that all milk samples were contaminated with AFM1 in which 91.8% of analyzed samples contained above permissible level of 0.05µg/l. Taddese *et al.*, (2020) investigated AFM1 in raw milk and reported that all 108 analyzed milk samples, were found to be contaminated by AFM1 with a mean value of 0.835µg/l. A study by Admasu *et al.* (2021), in South Gondar Zone to evaluate the contamination of AFM1 reported 99% of analyzed milk samples were contaminated with AFM1with values ranging from 0.031µg/l to 5.16µg/l and mean of 0.47µg/l. The result of the current study with 60.8% prevalence, 9.2% and 34.7% exceed the FDA and EU acceptable limit in raw milk is lower than the previous studies.

Mesfin *et al.* (2018), conducted study of AFM1 in raw milk in Holetta, Bishoftu and Hawassa. His result indicated mean AFM1 of 0.054µg/l. The mean AFM1 is lower than the mean (0.253µg/l of the current study. A study of AFM1 from raw milk of dairy farms in Injibara town of Amhara region by kassa (2020, indicated that 10 % of raw milk samples were contaminated with AFM1 is lower than the current study. This difference in the contamination levels can also be due to different cattle management systems, sources of feed and feed ingredients, and perhaps also feed storage conditions along the value chain.

The result of a study of AFM1 in raw milk from Kenya by Anyango, *et al.*, *(*2018), reported 26.4% of analyzed sample exceeded the limit of EU. Other studies in Kenya by Lindahi *et al*. (2018), indicated more than 50% of the samples exceeded EU maximum limit, but only three samples exceeded the USA acceptable limit. Kagera *et al.* (2019) reported Ninety nine percent

of the samples analyzed were contaminated with AFM1 with 64% of the samples exceeding the EU legal limit of 50ng/kg by analyzing raw milk samples collected from small-holder dairy farms using Enzyme-Linked Immunosorbent Assay (ELISA). Aflatoxin M1 was investigated from Fresh dairy milk from Small Individual Farms in Indonesia. Totally the incidence of AFM1 contamination is 21.15% none of these samples had AFM1 greater than the maximum level regulated in Indonesia (500ng/l or 0.5µg/l). Another study of AFM1 in Egypt by Esam *et al. (*2022), on raw milk in which 25.71% exceed the EU and Egyptian tolerance level and none of the samples exceeded the US tolerance level is lower than the current study. This difference in the contamination levels can also be due to different cattle management systems, sources of feed and feed ingredients, and perhaps also feed storage conditions along the value chain.

According to (Safwan, 2020) in Yemen, a total of 38 liquid milk samples (18 local, 20 imported) tested for AFM1. 68.42% were positive with an average contamination rate of 0.085µg/l from which 36.84% are above EU acceptable limit. The average contamination level of AFM1 is 0.085µg/l which is lower than the average of the current study with an average AFM1 contamination of 0.253µg/l.

Another study in India by Patyal *et al.(*2020), on 189 raw milk samples have a contamination rate of 58% with mean AFM1 of 0.917µg/l. 50.5% and 36.5% are above EU and Indian acceptable limit. The range of AFM1 is from 0.007- 13.1µg/l. The mean is by far higher than the current study. The maximum concentration of AFM1 (13.1µg/l) investigated was higher than the maximum of the current study.

Study from Pakistan by (Ahmed, *et al., (*2018) reported that 93% of the analyzed samples were contaminated by AFM1 and 69% of the samples exceeded the EU permissible limit (0.05 µg/L) which is by far much higher than the result of the current study. Another study on the occurrence of AFM1 in milk and milk products was conducted by (Bakirci, 2001) in Turkey indicated that 87.77% are positive of which 44.30% are above EU accepted limit. The result was also higher than the result of the current study. Detection of AFM1 in milk in Iraq using TLC, HPLC and ELISA. 38.5%, 50 % and 53.8% of milk samples were contaminated with

AFM1 respectively (Al-Mossawei *et al*., 2016). The reason for the difference of AFM1 in milk between Ethiopia and India could be due to the difference in feed type used in Ethiopia (wheat byproducts based feeds) and other African countries and India (by-products of maize and 55 groundnut based feeds) which are the most susceptible crops to AFB1 contamination as compared to wheat-based feeds (lewis *et al*., 2005)

Min *et al.* (2021) studied AFM1 on a total of 797 raw milk samples collected from ten provinces of Southern China. 11.80% were positive for AFM1, and none of them was above the Chinese and US legal limit, only 0.88% of samples were above the EU limit.

According to Ferrari *et al*.(2023),the European Union conducted AFM1 evaluation on a total of 95,882 samples of whole raw milk, collected in northern Italy between 2013 and 2021 using an ELISA (enzyme-linked immunosorbent assay) method. Only 667 milk samples 0.7% showed AFM1 values higher than the EU threshold limit of 50ng/L whereas all the other samples 99.3% complied with EU legislation. However, 0.4% of the total number of samples analyzed) showed AFM1 contamination between 40 and 50ng/L. The lower level of AFM1 contamination as well as the constant and very low percentage of positive samples out of the total number of samples analyzed, underline how monitoring programs are very effective in terms of control. Although Mediterranean and Middle Eastern countries have been faced in recent years with climate change, which increases extreme events (very high temperatures and exceptional amounts of rain) that favor AF contamination, the AFM1 concentration in milk and dairy products within the EU is usually at a very low level and well below the MRL (Prandini, 2009) thus indicating that strict controls and continuous monitoring of feedstuffs are effective.

A study in Ecuador by (Byron, *et al.,* 2020) on different provinces, on raw milk samples has a mean concentration of 0.077µg/l. All raw milk samples from various parts of Ecuador were positive for AFM1, Only 1.9% samples are above FDA and Ecuador limit of regulation (0.5µg/l) and 59.3% are incompliant to EU regulation. A study of AFM1from raw milk traded in Sao Polo, in Brazil by corrasin *et al.* (2022) has a prevalence of 12.5% from which 11.7% are above EU limit of tolerance but none of them were above the USA and the Brazil tolerance limit. The result of the current study is lower than the result of these studies

This variation in milk AFM1 level among studies/sites could be due to milk originating from different agro ecological conditions, applying different feeding practices, different climate conditions, different cattle management system, feed storage conditions, and different levels of awareness on feed and milk AFs. These variations could also be associated with different reasons like, season of sampling are associated with favorable conditions which are conducive for mold formation and growth. It could also be linked to the carryover rate of AFB1 as AFM1 in milk which varies widely among animals, 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 (Ayhan, 2010).

Another reason could be due to the analytical methods used for the analysis of AFM1. Previously most researches are analyzed by Enzyme Linked Immuno Sorbent Assay but currently most researchers are using latest equipment like HPLC to analyze AFs. Methods of analysis for determining AFM1 in milk should be able to detect traces of AFM1 at the level of ng/kg. This performance criterion has been accomplished using the immuno affinity column purification step, followed by LC separation and fluorescence detection. LC is a method that provides good sensitivity, high dynamic range and versatility. Detection by LC is usually made by FLD, UV absorption or mass spectrometry detection (Espinosa-Calderón *et al*., 2011).

If we want to see the prevalence of AFM1 of raw milk from dairy farms and collection centers individually, the analysis of 25 samples of raw milk from milk collection sites in Adama town revealed a prevalence of 96% (24), but 96% (24) of the samples were infected with AFM1. 20% (5) and 72% (18) of these contaminated samples were above the USA/ES and EU acceptable levels, respectively. Raw milk gathered from collection centers had AFM1 concentrations as low as 0.012µg/l and as high as 8µg/l. According to table 8 above, the average and standard deviation of AFM1 for raw milk from collection points are 0.85µg/l and 1.91µg/l, respectively.

This study examined 95 milk samples in total from dairy farms, and a prevalence of 51.6% (49) AFM1was detected. From which 6 (6.3%) and 33 (34.7%) milk samples exceeded the FDA /ESA and EU permitted levels, respectively. AFM1 concentrations in milk samples from dairy farms ranged from 0.016µg/l to 0.655µg/l, respectively. As shown in Table 7, the average and standard deviation of AFM1 for raw milk from the dairy farms were 0.096µg/l and 0.16µg/l, respectively.

AFM1 contamination of raw milk from dairy farms and collection centers differed statistically significantly (p value = 0.000). Aflatoxin M1 was present in raw milk samples from milk collection centers at a prevalence of 96% versus 51.6% from dairy farms. Similar results were found in an Iranian study that revealed the concentration of AFM1 in raw milk samples from dairy producers was much lower than that in samples from collectors and processors (Hashemi, 2019). This variation could be due to large volume mixing of different levels of AFM1contaminated raw milk from different producers, through formal and informal means, at the milk collection centers, thereby increasing the level of AFM1 in mixed raw milk. In Ethiopia, 95% of milk is channeled through the informal market, where dairy farmers sell to neighbors, small unions, cooperatives and retails (TAM Consult, 2008).

Another possible reason for the high contamination rate of AFM1in raw milk of collection centers obtained in this study may be due to the fact that dairy animals kept in local dairy farms were fed with compound rations stored under inadequate conditions, which can be contaminated with AFs. Hot and humid climatic conditions are very conducive for fungal invasion, growth and production of mycotoxins including AFs in food and feed commodities.
The unorganized farms dominating the dairy industry are mainly owned by small or marginal farmers which supply raw milk for the collection centers. Kumar *et al. (*2011) reported that more than 75% of milk production and marketed surplus of milk is contributed by small dairy farm holders. Because of poor farming knowledge and economic constraints, animal feed storage and farm management conditions in small scale farms are often not appropriate which may lead to occurrence of AFs in the dairy animal's feedstuff and raw milk (Kumar *et al. (*2011).

Similar to the current study Hashemi, (2016), evaluated AFM1 levels in 168 samples of raw milk (135 samples and 33 samples from bulk tanks of farms and milk collection centers, respectively) and 12 samples of pasteurized milk in Fars province, Southern Iran. AFM1 was found in 55.56% of the samples with a mean concentration of 21.31ng/L. The concentration of AFM1 in raw milk samples from farms was lower than that in samples from collection centers and pasteurized milk. The raw milk produced in smallholder farms is collected and pooled with other milk during cooling in milk collection centers and then transported to pasteurization plants in Iran. No testing for contamination with AFM1 is done prior to receiving raw milk in milk collection centers. This leads to mixing of raw milk with different AFM1 levels, and subsequently elevating the level of contamination in transported milk to pasteurization plants

Questionnaire data on dairy farm owners' feed management practice, which are risk factors for the development of AFs, was also gathered. In order to determine whether there is a statistically significant correlation between these risk factors and the development of AFM1, we attempted to link the response of dairy farm owners with the outcome of the laboratory test for AFM1. Logistic regression analysis showed that farm management practices like storage time, storage place, moisture control and type of feed, are significantly associated with the occurrence of AFM1 in raw milk.

Due to increased pressure on land for human settlement and absence of resources for farmers to run large scale dairy farming units, zero-grazing is the production system of choice for farmers in urban and peri urban areas. In this study, majority of famers practice zero-grazing, thus relying more on commercial pasture, fodder and concentrates with only a few making their own on farm formulations.

Being zero grazing has a significant effect on contamination level of AFM1. In this study from 95 respondents 15.8% and 84.2% responded that they use mixed grazing and zero grazing (intensive management) respectively from which only 6.7% and 60% of them are positive for AFM1 respectively during laboratory analysis. In this study high contamination level of AFM1 was recorded in milk originated from farmers who use zero grazing and low AFM1

contamination in milk is observed from farmers who practice mixed grazing practice. This difference is statistically significant in which p-value of 0.010 is less than 0.05.

Here, the reason probably during zero grazing system concentrate feeding is more applicable and dairy farmers who feed concentrate for their cattle increase the probability of having AFM1 in the milk sample. A Kenyan study revealed that feeding concentrates are more likely to have a high level of AFM1 than others (Anyango *et al*., 2018). Readymade concentrate feedstuffs constituted the major portion of animal's diet. Concentrate feeds are more vulnerable to the attack of molds and there is a high possibility of presence of AFB1 in these commodities. Urban dairy farmers may practice concentrate feeding because of the inaccessibility of other feeding mechanisms, storing animal feed in a restricted, and closed room, keep cows within a confined grazing area that may favor, and facilitate mold growth and contamination of feed by AFM1(Soler *et al*., 2010). Furthermore, urban and peri urban countries may lack diversification of food items which may balance the susceptible and unsusceptible animal feeds as against monolithic susceptible animal feed (Anthony *et al*., 2016)

A study by Patyal *et al.,* (2020), in India indicated that dairy industry is mainly dominated by unorganized farms owned by small scale dairy farmers and it has been observed during his study that due to poor socioeconomic conditions of small scale farmers, the household cereals such as maize, wheat which were left unconsumed by humans, often finds their way into dairy animal's diet. High AFs contamination of such cereals has been reported from India by several investigators in previous studies (Toteja, *et al.,* 2006*)*

Households that have no animal feed storage facility are more likely to have higher levels of AFM1 in milk when compared to households that have a storage facility. Feed that is not kept on raised and ventilated platforms, stored indoors, or in long-term storage conditions may create conducive environment for the accumulation of molds (Dawit *et al.,* 2016). With no storage or improper storage conditions such as high humidity and temperature, fungal growth is favored, and there is an increased risk of mycotoxin in milk (Chaisri *et al.,* 2017). The feedstuff which was stored under poor storage conditions (high relative humidity and

temperature, poor ventilation) had a high probability of being contaminated with AFs. The high risk of AFM1 contamination in dairy farms storing dairy animal's feed under poor storage conditions for longer durations in this study might be explained by the high humidity within storage area/room, which is favorable for fungal growth and increased AFB1 production. (Erbez *et al.,* 2010).

During our questionnaire survey, we found that 46.3%, 31.6%, and 22.1% store their feed in the house, in shade, and on an open space, respectively. The laboratory results of this study indicated that 36.3%, 53.3%, and 80.9% of milk samples taken from farms who store their feed in house, in shade and on open space respectively were contaminated with AFM1. There is a statistically significant difference ($p = 0.00$) between these farms in the occurrence of AFM1 because of their storage locations. The current study is also different from Sisay (2019). He reported that 59%, 24%, and 13% of them store their feed in the house, in shade, and in open space, respectively.

The duration of storage also has a statistically significant effect ($p = 0.010$) on the occurrence of AM1. In this study, milk from those farms that store their feed for more than one week has a higher AFM1 contamination rate than milk from those farms that store their feed for one week. This is supported by laboratory analysis results. From 95 farms, 65.3% store their feed for more than one week, of which 62.9% of the milk is contaminated with AFM1, whereas 34.7% of the farms store their feed for one week, and 30.3% of the milk from these farms is contaminated with AFM1. The current study is different from a study by Sisay (2019) in selected kebeles of Bishoftu and Adama. According to this study who reported that around 80% of the dairy and beef farms do not store feed for long time because of shortage of appropriate storage place for the feed.

The feedstuff which was stored under poor storage conditions i.e. high relative humidity and temperature, poor ventilation for long time had a high probability of being contaminated with AFs. The high risk of AFM1 contamination in dairy farms storing dairy animal's feed under poor storage conditions for longer durations in this study might be explained by the high humidity within storage area/room, which is favorable for fungal growth and increased AFB1

production (Erbez, 2010).

Unless it is effectively controlled on the farm, moisture is one of the biggest risk factors for the formation of AFs in feed. According to our interview, 52.6% of dairy farmers use ventilation, pallets, concrete floors, crates and kept away the feed from walls and roofs to reduce moisture in feed storage facilities. Laboratory testing on samples from these farms revealed that 32% of them are contaminated with AFM1, which is less than 71.1% contamination rate of AFM1 for facilities that did not control moisture.

There is statistically significant difference between those, which control and did not control feed moisture for the occurrence of AFM1 in milk. This is consistent with the findings by Strosnider *et al.* (2006), who reported that it is advisable to keep the grains from contact with the earth, raising them on wooden pallets or on concrete floor and ensuring adequate ventilation in a storage facility helps to prevent an increase in moisture content, insect and rodent infestation during storage, and is a critical measure against AF contamination.

The findings by Hawkins *et al.* (2005), confirms that high levels of humidity, temperature and poor aeration during storage are important factors that may contribute to AF contamination. In another study, Smith *et al*. (1985), further emphasizes that AF contamination can occur when food commodities are stored under high moisture and temperature conditions.

6. CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

Aflatoxin M1 occurrence in Adama is very high and it is necessary to make planned monitoring of milk and make efforts to reduce the AFB1 content in the feed of dairy cattle and, therefore, AFM1 in raw milk. In the present study 60.8% of raw milk samples collected from dairy farms and collection centers in and around adama town were contaminated with AFM1with different degrees. From contaminated milk 9.2% is beyond the acceptable limit of ES and unfit for human consumption and 42.5% were found to be above the regulatory limits set by EU. KAP survey result of this study indicated that farm management practices like feed source, feed storage house, storage time and moisture are significant risk factors for the occurrence of AFM1 in raw milk. The level of contaminated milk with AFM1 reported in this study should be a wakeup call for stringent monitoring of raw materials and feed samples to prevent cattle exposure to AFs contaminated feeds which would lead to excretion of AFM1 in milk and eventually causing human exposure through consumption of contaminated milk.

6.2. Recommendations

- Increasing controls and creating awareness for dairy farmers on public health problems caused by AFM1 and how to produce quality animal feed and milk by value chain actors.
- There is a need to conduct risk assessment studies with dairy product consumers in different age groups.
- Exploring different mitigation strategies to control AFM1 in milk in urban and peri-urban areas is essential.
- Feed ingredients and finished products should be thoroughly monitored to prevent cattle exposure to contaminated feeds which would lead to excretion of AFM1 in milk causing human exposure through consumption.

7. REFERENCES

AOAC official method. (2002): Aflatoxin M1 in Liquid milk Immuno affinity Column by Liquid Chromatography. AOAC international.

Abdel-Wahhab, M. A., Omara, E. A., Abdel-Galil, M. M., Hassan, N. S., Nada, S. A., Saeed, A., & ElSayed, M. M. (2007). Zizyphus spina-christi extract protects against aflatoxin B1-intitiated hepatic carcinogenicity. *African Journal of Traditional, Complementary and Alternative Medicines*, ***4***(3): 248.

Admasu, F. T., Melak, A., Demissie, B., Yenew, C., Habtie, M. L., Bekele, T. T. & Dejenie, T. A. (2021). Occurrence and associated factors of aflatoxin M1 in raw cow milk in South Gondar Zone, North West Ethiopia. *Food Science & Nutrition*, ***9***(11): 6286-6293.

Ahmad, M., Awais, M., Ali, S. W., Ali Khan, H. A., Riaz, M., Sultan, A. & Ishtiaq Chaudhry, A. (2019). Occurrence of Aflatoxin M1 in raw and processed milk and assessment of daily intake in Lahore, Multan cities of Pakistan. *Food Additives & Contaminants: Part B*, ***12***(1): 18-23.

Akram, M., Shahab-Uddin, A. A., Usmanghani, K. H. A. N., Hannan, A. B. D. U. L., Mohiuddin, E., & Asif, M. (2010). Curcuma longa and curcumin. A review article. *Rom J Biol Plant Biol*. ***55***(2): 65-70.

Al-Mossawei, M. T., Al-Zubaidi, L. A., Hamza, I. S., & Abduljaleel, S. Y. (2016). Detection of AFM1 in Milk and Some Dairy Products in Iraq Using Different techniques. *Advances in Life Science and Technology*, ***41***:74-81.

Ames, I. A. (1989). Mycotoxins, economic and health risks. *CAST (Council of Agricultural Science and Technology), Task force Rep. 116*.

Anfossi, L., Baggiani, C., Giovannoli, C., & Giraudi, G. (2011). Occurrence of Aflatoxin M1 in Dairy Products, Aflatoxin-Detection. *Measurement and Control, Dr Irineo Torres-Pacheco (Ed.)*.

Anthony, M. H., Ojochenemi, A. D., Mulunda, M., Oriyomi, S. T., Jideofor, N. F., Tunde, O. & Isah, A. (2016). Aflatoxin M1 in breast milk, cow milk and milk products in Minna, Nigeria and their predisposing factors. *Biochemistry & analytical biochemistry*.***5***(4): 1-6.

Anyango, G., Mutua, F., Kagera, I., AndangO, P., Grace, D., & Lindahl, J. F. (2018). A survey of aflatoxin M1 contamination in raw milk produced in urban and peri-urban areas of Kisumu County, Kenya. *Infection Ecology & Epidemiology*, ***8***(1): 1547094.

Applebaum R. S., R. E. Brackett D. W. Wiseman and E. H. Marth. (1982). Aflatoxin: toxicity to dairy cattle and occurrence in milk and milk products-a review. *Journal of Food Protection,* **45**:752.

APVDFQAC (2019). Standard operating Procedure (SOP) for HPLC Determination of fatty Feed Aflatoxin using *UVE* (QMS-PHL-TXA-SOP-09).

Ayoub M., El-Far A., Taha N., Korshom M., Mandour A., Abdel-Hamid H. (2011). The biochemical protective role of some herbs against aflatoxicosis in ducklings: I. *Turmeric. Lucrări Ştiinţ Univ Ştiinţ Agricole Med Veterinară Ser Zootehnie,* **55**:150-9.

Bakirci, I. (2001). A study on the occurrence of aflatoxin M1 in milk and milk products produced in Van province of Turkey. *Food control*, ***12***(1): 47-51.

Balina, A., Kebede, A., & Tamiru, Y. (2018). Review on Aflatoxin and its Impacts on Livestock. *Journal of Dairy and Veterinary Sciences*, ***6***(2): e555685.

Bankole, S. A., & Adebanjo, A. (2003). Mycotoxins in food in West Africa: current situation and possibilities of controlling it. *African journal of Biotechnology* ***2***(9): 254-263.

Benkerroum, N. (2020). Aflatoxins: Producing-molds, structure, health issues and incidence in Southeast Asian and Sub-Saharan African countries. *International journal of environmental research and public health*, ***17***(4): 1215.

Benkerroum, N. (2020). Chronic and acute toxicities of aflatoxins: Mechanisms of action. *International journal of environmental research and public health*, ***17***(2): 423.

Bennett, J.W. (1987). Mycotoxins, mycotoxicoses, mycotoxicology and mycopathologia. *Mycopathologia*, *100*: 3-5.

Bennett, J.W. and Klich, M. (2003). Mycotoxins," *Clinical Microbiology Reviews*, vol. **16**: pp. 497–516.

Bianchini, A. and Bullerman, L.B. (2010). Biological control of molds and mycotoxins in foods. Mycotoxin prevention and control in agriculture. ACS symposium series. Washington, DC: *American Chemical Society*.

Blout W.P. (1961). Turkey "X" disease.*Turkeys*, **9**:52: 55-58.

Boutrif, E. (1998). Prevention of aflatoxin in pistachios. *Food Nutrition and Agriculture*, pp.32-37.

Bozoğlu, F. (2009). Different mycotoxin inactivation applications and their inactivation mechanisms. *Zbornik Matice srpske za prirodne nauke*, (**117**): 27-35.

Bryden, W. L. (2007). Mycotoxins in the food chain: human health implications. *Asia Pacific journal of clinical nutrition*. ***16*** (S1): 95-101.

Bukari, N., Kwofie, M. K., & Adeboye, O. (2020). Aflatoxin M1 (*Aspergillus parasiticus, flavus*) occurrences in milk and milk products and its possible health effects. *Advances in Microbiology*, ***10***(10): 509.

Bullerman, L. B., & Bianchini, A. (2007). Stability of mycotoxins during food processing. *International journal of food microbiology*, ***119***(1-2): 140-146.

Buszewska-Forajta, M. (2020). Mycotoxins, invisible danger of feedstuff with toxic effect on animals. *Toxicon*, ***182***:34-53.

CAC (2017), Codex General Standard for Contaminants and Toxins in Food and Feed. Codex Standard 193-1995.

Cavaliere, C., Foglia, P., Guarino, C., Marzioni, F., Nazzari, M., Samperi, R., & Laganà, A. (2006). Aflatoxin M1 determination in cheese by liquid chromatography–tandem mass spectrometry. *Journal of Chromatography A*, ***1135***(2): 135-141.

CES (Compulsory Ethiopian Standard 278) (2021). Raw whole cow milk – Specification. Second Edition, Published by Ethiopian Standards Agency © ESA

Chaisri, W., Mongkon, W., Sugita-Konishi, Y., Van Dam, D., Huntley, I., & Suriyasathaporn, W. (2017). Feed and feed storage factors in relation to aflatoxin M1 contamination in bulk milk of smallholder dairy farms. *JSM mycotoxins*, ***67***(2): 85-88.

Chala, A., Taye, W., Ayalew, A., Krska, R., Sulyok, M., & Logrieco, A. (2014). Multimycotoxin analysis of sorghum (Sorghum bicolor L. Munch) and finger millet (Eleusine coracana L. Garten) from Ethiopia. *Food Control*, ***45***: 29-35.

Codex Alimentarius Commissions (2001). *Comments submitted on the draft maximum level for Aflatoxin M1 in milk*. Codex committee on food additives and contaminants 33rd sessions, The Hauge, The Netherlands.

Corassin, C. H., Borowsky, A., Ali, S., Rosim, R. E., & de Oliveira, C. A. F. (2022).

Corassin, C. H., Borowsky, A., Ali, S., Rosim, R. E., & de Oliveira, C. A. F. (2022).

Occurrence of Aflatoxin M1 in Milk and Dairy Products Traded in São Paulo, Brazil. An Update. *Dair**y, 3***(4: 842-848.

CSA: Addis Ababa, Ethiopia. Volume II, p. 98. 37. ES ISO 707; Milk and Milk Products-Guidance on Milk Sampling (2012): 1st ed. *Ethiopian Standards Agency*, pp. 1–45.

Dalvi, R. R. (1986). An overview of aflatoxicosis of poultry: Its characteristics, prevention and reduction. *Veterinary research communications*, ***10***(1): 429-443.

Dashti, B., Al-Hamli, S., Alomirah, H., Al-Zenki, S., Abbas, A. B., & Sawaya, W. (2009). Levels of aflatoxin M1 in milk, cheese consumed in Kuwait and occurrence of total aflatoxin in local and imported animal feed. *Food Control*, ***20***(7): 686-690.

Demissie, N. (2018). A review of aflatoxin: occurrence, prevention, and gaps in both food and feed safety. *Journal of Nutritional Health & Food Engineering*, ***8***(2).

Desalegn, SG. (2018): Milk Value Chain Analysis in Sebeta District, Central high land of Ethiopia. *Food Sci Qual Manag.***75**:51–66.

Deveci, O. (2007). Changes in the concentration of aflatoxin M1 during manufacture and storage of White Pickled cheese. *Food control*, ***18***(9): 1103-1107.

Devegowda, G., M.V.L.N. Raju and H.V.L.N. Swamy. (1998). Mycotoxins: Novel solution for their counteraction, Feedstuffs, **70** (50): 12-15

El Khoury, A., Atoui, A., & Yaghi, J. (2011). Analysis of aflatoxin M1 in milk and yogurt and AFM1 reduction by lactic acid bacteria used in Lebanese industry. *Food control*, ***22***(10): 1695-1699.

Erbez, M., Falta, D., & Chládek, G. (2010). The relationship between temperature and humidity outside and inside the permanently open-sided cow's barn. *Acta Universitatis agriculturae et silviculturae Mendelianae Brunensis*, ***58***(5):91-96.

Esam, R. M., Hafez, R. S., Khafaga, N. I. M., Fahim, K. M., & Ahmed, L. I. (2022). Assessment of aflatoxin M1 and B1 in some dairy products with referring to the analytical performances of enzyme-linked immunosorbent assay in comparison to high-performance liquid chromatography. *Veterinary World*, ***15***(1): 91.

Espinosa-Calderón, A.,Contreras-Medina, L. M., Muñoz-Huerta, R. F., Millán-Almaraz, J. R., González, R. G. G., & Torres-Pacheco, I. (2011). Methods for detection and quantification of aflatoxins. *Aflatoxins: detection, measurement and control. New York: InTech*, 109-128.

Ethiopia Capital News (2016). Ethiopian Capital News Letter Ethiopia to Use Aflasafe Technology to Reduce Aflatoxin in Red Pepper. Available online.

European Union (2006). Commission Regulation (EU) No 165/2010 of 26 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. *Journal of European Union,* **50**: 8-12.

FAO (2011): Worldwide regulations for micotoxins in food and in feed in 2003. *FAO.* Food and Nutrition Paper. 14*8*.

Ferrari, L., Rizzi, N., Grandi, E., Clerici, E., Tirloni, E., Stella, S., & Pinotti, L. (2023). Compliance between Food and Feed Safety: Eight-Year Survey (2013–2021) of Aflatoxin M1 in Raw Milk and Aflatoxin B1 in Feed in Northern Italy. *Toxins*, ***15*** (3): 168.

Galvano, F., Galofaro, V., & Galvano, G. (1996). Occurrence and stability of aflatoxin M1 in milk and milk products: a worldwide review. *Journal of Food protection*, ***59***(10): 1079-1090.

Goda, Y., Akiyama, H., Otsuki, T., Fujii, A., & Toyoda, M. (2001). Application and improvement of aflatoxin analysis in foods using a multifunctional column and HPLC. *Shokuhin Eiseigaku zasshi. Journal of the Food Hygienic Society of Japan*, ***42***(1): 56-62.

Goldblatt, Ed. Academic Press, New York, 1969.xiv+ 474 pp., illus. $12.50. Food Science and Technology, vol. 7. *Science*, ***168***(3936): 1193-1193.

Goyal, R. K. (1991). Prevention and control of mycotoxins in food grains in India.

Hashemi, M. (2016). A survey of aflatoxin M1 in cow milk in Southern Iran. *Journal of food and drug analysis*, ***24***(4): 888-893.

Hawkins, L. K., Windham, G. L., & Williams, W. P. (2005). Effect of different postharvest drying temperatures on Aspergillus flavus survival and aflatoxin content in five maize hybrids. *Journal of food protection*, *68*(7), 1521-1524.

Henshall, J. D. (2012). Food safety and standards authority of India ministry of health and family welfare government of India New Delhi. *Manual of Methods of Analisis of Foods Fruit and Vegetable Products*, *5*, 1-59.

Hussain, I. (2011). Aflatoxin measurement and analysis. *Aflatoxins–Detection, Measurement*

and Control. 1st ed. Rijeka, Croatia: InTech, 129-146.

IARC (1993): Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, Lyon, France. 56: 245-395.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. International Agency for Research on Cancer & World Health Organization. (2002). *Some traditional herbal medicines, some mycotoxins, naphthalene and styrene* (Vol. 82). World Health Organization.

Iqbal, S. Z., Jinap, S., Pirouz, A. A., & Faizal, A. A. (2015). Aflatoxin M1 in milk and dairy products, occurrence and recent challenges: A review. *Trends in Food Science & Technology*, ***46***(1): 110-119.

Jaimez, J., Fente, C. A., Vazquez, B. I., Franco, C. M., Cepeda, A., Mahuzier, G., & Prognon, P. (2000). Application of the assay of aflatoxins by liquid chromatography with fluorescence detection in food analysis. *Journal of Chromatography A*, ***882*** (1-2): 1-10.

Jaiswal, P., Jha, S. N., Kaur, J., Borah, A., & Ramya, H. G. (2018). Detection of aflatoxin M1 in milk using spectroscopy and multivariate analyses. *Food Chemistry*, ***238***:209-214.

Josephs, R. D., Ulberth, F., Van Egmond, H. P., & Emons, H. (2005). Aflatoxin M1 in milk powders: Processing, homogeneity and stability testing of certified reference materials. *Food additives and contaminants*, ***22***(9): 864-874.

Jouany, J. P., Yiannikouris, A., & Bertin, G. (2009). Risk assessment of mycotoxins in ruminants and ruminant products. *Options mediterranéennes, A*, ***85***: 205-224.

Kagera, I., Kahenya, P., Mutua, F., Anyango, G., Kyallo, F., Grace, D., & Lindahl, J. (2019). Status of aflatoxin contamination in cow milk produced in smallholder dairy farms in urban and peri-urban areas of Nairobi County: a case study of Kasarani sub county, Kenya. *Infection ecology & epidemiology*, ***9***(1): 1547095.

Karimi Dehcheshmeh, B., Shakerian, A., & Rahimi, E. (2021). Evaluation of aflatoxin M1 and heavy metal in raw materials and infant formula produced in Pegah dairy plants, Iran. *Journal of Chemical Health Risks*, ***11***(1): 55-62.

Kassa, A., Talema, A., & Ketsela, G. (2020). Determination of Aflatoxin M1 in Raw Cow's Milk by Using HPLC-FLD, in Injibara Town, Awi Zone, Amhara, Ethiopia.

Ketney, O., Santini, A., & Oancea, S. (2017). Recent aflatoxin survey data in milk and milk

products: A review. *International Journal of Dairy Technology*, ***70(***3): 320-331.

Kumar, A., Staal, S. J., & Singh, D. K. (2011). Smallholder dairy farmers' access to modern milk marketing chains in India. *Agricultural Economics Research Review*, ***24***(347-2016-16969): 243-254.

Lindahl, J. F., Kagera, I. N., & Grace, D. (2018). Aflatoxin M1 levels in different marketed milk products in Nairobi, Kenya. *Mycotoxin Research*, ***34***(4): 289-295.

Liu, Y., & Wu, F. (2010). Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. *Environmental health perspectives*, *118*(6): 818-824.

Marchese, S., Polo, A., Ariano, A., Velotto, S., Costantini, S., & Severino, L. (2018). Aflatoxin B1 and M1: Biological properties and their involvement in cancer development. *Toxins*, ***10***(6):214.

Matabaro, E., Ishimwe, N., Uwimbabazi, E., & Lee, B. H. (2017). Current immunoassay methods for the rapid detection of aflatoxin in milk and dairy products. *Comprehensive reviews in food science and food safety*, ***16***(5): 808-820.

Merwe, D. Van Der, Birkelo, C., & Zereyesus, Y. (2019). A survey of mycotoxins in livestock feeds in Ethiopia. 3.

Mesfin, R., Assefa, G., & Assefa, F. (2018). Determination of aflatoxin in dairy feeds and milk in some selected areas of Ethiopia. *Food and Environment Safety Journal*, ***17***(3).

Milićević, D. R., Škrinjar, M., & Baltić, T. (2010). Real and perceived risks for mycotoxin contamination in foods and feed. challenges for food safety control. *Toxins*, ***2***(4): 572-592.

Min, L., Tong, X., Sun, H., Ding, D., Xu, B., Chen, W., & Li, D. (2021). Aflatoxin M1 contamination in raw milk and its association with herd types in the ten provinces of Southern China. *Italian Journal of Animal Science*, ***20***(1): 1562-1567.

Murshed, S. (2020). Evaluation and assessment of aflatoxin M1 in milk and milk products in Yemen using high-performance liquid chromatography. *Journal of Food Quality*, 1-8.

Mutegi, C. K., Cotty, P. J., & Bandyopadhyay, R. (2018). Prevalence and mitigation of aflatoxins in Kenya (1960-to date). *World Mycotoxin Journal*, ***11***(3): 341-357.

Nguefack, J., Leth, V., Zollo, P. A., & Mathur, S. B. (2004). Evaluation of Five Essential Oils From Aromatic Plants of Cameroon for Controlling Food Spoilage and mycotoxin producing fungi. *International Journal of Food Microbiology*, ***94***(3):329-334.

Niessen, L. (2007). PCR-based diagnosis and quantification of mycotoxin producing fungi. *International journal of food microbiology*, ***119***(1-2):38-46.

Nishimwe, K., Bowers, E., Ayabagabo, J. D. D., Habimana, R., Mutiga, S., & Maier, D. (2019). Assessment of aflatoxin and fumonisin contamination and associated risk factors in feed and feed ingredients in Rwanda. *Toxins*, ***11***(5): 270.

Nogaim, Q. A., Al-Dalali, S., Al-Badany, A., & Farh, M. (2014). Scientia Research Library. *Journal of Applied Chemistry*, ***2***(4): 13-18.

Owens, R. G. (1970). Carcinogens: Aflatoxin. Scientific Background, Control, and Implications. Leo A.

Palumbo, P. and Lima N. (2010). How will climate change affect mycotoxins in food Food Research International. **43**(7):1902-1914.

Patyal, A., Gill, J. P. S., Bedi, J. S., & Aulakh, R. S. (2020). Potential risk factors associated with the occurrence of aflatoxin M1 in raw milk produced under different farm conditions. *Journal of Environmental Science and Health, Part B*, ***55***(9):827-834.

Polak-Śliwińska M.(2020). Determination of aflatoxin M1 contamination levels in milk and milk products by HPLC-FLD. Technol Prog food Process.110–6.

Rahmani, A., Jinap, S., & Soleimany, F. (2009). Qualitative and quantitative analysis of mycotoxins. *Comprehensive reviews in food science and food safety*, ***8***(3):202-251.

Reddy, K. R. N., Nurdijati, S. B., & Salleh, B. (2010). An overview of plant-derived products on control of mycotoxigenic fungi and mycotoxins. *Asian Journal of Plant Sciences*, ***9***(3): 126.

Sani, AM. Nikpooyan, H. (2013). Determination of aflatoxin M1 in milk by high performance liquid chromatography in Mashhad (north east of Iran). Toxicol Ind Health. **29**(4):334–8.

Santini, A. and Ritieni, A. (2013). Aflatoxins: Risk, Exposure and Remediation. *Journal of open science. 52866.*

Sapsford, K. E., Taitt, C. R., Fertig, S., Moore, M. H., Lassman, M. E., Maragos, C. M., & Shriver-Lake, L. C. (2006). Indirect competitive immunoassay for detection of aflatoxin B1 in corn and nut products using the array biosensor. *Biosensors and bioelectronics*, ***21***(12): 2298-2305.

Schiefer (1990). Mycotoxicosis of domestic animals and their diagnosis. Physio, 987-990

Senerwa, D. M., Anadem Mtimet, A. J. Sirma, J. Nzuma, Erastus K. Kang'ethe, Johanna F. Lindahl, and Delia Grace.(2016) "Direct market costs of aflatoxins in Kenyan dairy value chain."

Sharma H, Jadhav VJ, Garg SR.(2019). Aflatoxin M1 in milk in Hisar city , Haryana , India and risk assessment. Food Addit Contam Part B 1–5.

Shinha, K. K., & Bhatnagar, D. (1998). *Mycotoxins in agriculture and food safety*. CRC Press.

Soler, T., Hoogenboom, G., Olatinwo, R., Diarra, B., Waliyar, F., & Traore, S. (2010). Peanut contamination by Aspergillus flavus and aflatoxin B1 in granaries of villages and markets of Mali, West Africa. *Journal of Food, Agriculture & Environment*, ***8***(2): 195-203.

Stepman, F. (2018). Scaling-up the impact of aflatoxin research in Africa. The role of social sciences. Toxins.10 (136).

Strosnider, H., Azziz-Baumgartner, E., Banziger, M., Bhat, R. V., Breiman, R., Brune, M. N. ... & Wilson, D. (2006). Workgroup report: public health strategies for reducing aflatoxin exposure in developing countries. *Environmental health perspectives*, *114*(12), 1898-1903.

Szonyi, B., Dawit, G., D.,Tegegne, A., Jean Hanson,J. and Delia Grace,D. *(2015).* Aflatoxin M1 contamination of milk in the Greater Addis Ababa milk shed, Ethiopia.1st African Symposium on Mycotoxicology. Livingstone, Zambia.26-28

Tadesse, S., Berhanu, T., & Woldegiorgis, A. Z. (2020). Aflatoxin M1 in milk and milk products marketed by local and industrial producers in Bishoftu town of Ethiopia. *Food control*, ***118***: 107386.

TAM Consult (2008). Dairy Investment Opportunities in Ethiopia; Study on Dairy Investment Opportunities in Ethiopia; SNV Netherlands Development Organization: The Hague, the Netherlands, pp. 1–59. 17.

Thembo, K. M., Vismer, H. F., Nyazema, N. Z., Gelderblom, W. C. A., & Katerere, D. R. (2010). Antifungal activity of four weedy plant extracts against selected mycotoxigenic fungi. *Journal of applied microbiology*, ***109***(4): 1479-1486.

Thrasher, JD. (2012). Aflatoxicosis in animals. Aflatoxins and Health.

Thrusfield, M. (2005): Veterinary Epidemiology. In *Equine Internal Medicine: Second Edition* (3rd ed.)

Toteja, G. S., Mukherjee, A., Diwakar, S., Singh, P., Saxena, B. N., Sinha, K. K., & Parkar, S. (2006). Aflatoxin B1 contamination in wheat grain samples collected from different geographical regions of India: a multicenter study. *Journal of food protection*, ***69***(6):1463-1467.

USAID & DANYA (2012). *Aflatoxin:* A synthesis of the research in health, agriculture, and trade. 3390/toxins12100648.

USAID (2012): Aflatoxin: A Synthesis of the Research in Health, Agriculture and Trade. Feed the Future: The Office of Regional Economic Integration USAID East Africa Regional Mission Nairobi, Kenya.10-15.

Vosough, M., Bayat, M., & Salemi, A. (2010). Matrix-free analysis of aflatoxins in pistachio nuts using parallel factor modeling of liquid chromatography diode array detection data. *Analytica chimica acta*, ***663***(1): 11-18.

WHO (2000). Hazardous Chemicals in Humans and Environmental Health: International Program on Chemical safety, Geneva, Switzerland. World Health Organization. 7-9.

WHO (2005). Public health Strategies for Preventing Aflatoxin exposure. Work group report for the international Mycotoxin Workshop.World Health Organization, Geneva, Switzerland. 1- 26.

Widiastuti, R., & Anastasia, Y. (2018). Aflatoxin M1 in fresh dairy milk from small individual farms in Indonesia.

Williams, J. H., Phillips, T. D., Jolly, P. E., Stiles, J. K., Jolly, C. M., & Aggarwal, D. (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *The American journal of clinical nutrition*, ***80***(5): 1106-1122.n Geneva, Switzerland.

Wolde, M.(2017). Effects of aflatoxin contamination of grains in Ethiopia. *International Journal of Agricultural Science*, ***7***(4): 1298-308.

World Health Organization. (2015). *WHO estimates of the global burden of foodborne diseases: foodborne disease burden epidemiology reference group 2007-2015*. World Health Organization.

Wu, F., & Khlangwiset, P. (2010). Health economic impacts and cost-effectiveness of aflatoxin-reduction strategies in Africa: case studies in bio control and post-harvest interventions. *Food Additives and Contaminants*, ***27(***4):496-509.

Wu, F., Narrod, C., Tiongco, M., & Liu, Y. (2011). The health economics of aflatoxin: Global burden of disease. International Food Policy Research Institute.

Yohannes, B., Wondossen, A. & Anteneh, G. (2018). Analysis to ascertain the determination for aflatoxin contamination of milk and feeds from Gurage zone, Ethiopia. *International journal of Agricultural Research*, ***13***(1): 1-11.

Yousef, A., & Marth, E. (1989). Stability and degradation of aflatoxin M1. Mycotoxins in dairy products, edited by HP van Egmond.

Zain, M. (2011). Impact of mycotoxins on human and animals. *Journal of Saudi Chemical Society*, **15**:129-144.

Zebib, H., Abate, D., & Woldegiorgis, A. Z. (2022). Aflatoxin M1 in raw milk, pasteurized milk and cottage cheese collected along value chain actors from three regions of Ethiopia. *Toxins*, ***14***(4):276.

8. ANNEXES

Annex 1: Consent of Agreement

To effectively attain the objective of this research, I am requesting your help. For your participation in the study no payment will be granted or has no any special privilege to you. All information you give will be kept confidential and won't be accessible to any third party; your name won't be registered on the question sheet so that you will not be identified for any reason. You have the right not to participate from the beginning, or you may stop participating at any time after starting the participation. However, your honest answers to these questions will help me in better understanding of AFs knowledge, practice and associated factors and will eventually help in designing and implementing appropriate interventions to alleviate related problems.

Will you be willing to participate? ◯ Yes ◯ No

Informed consent: I have read this form or it has been read to me in the language I understand all conditions stated above. Therefore, I am willing to participate in this study.

Signature _____Date_____

Data collector name signature

Questionnaires

Part 1. Socio Demographic Characteristics

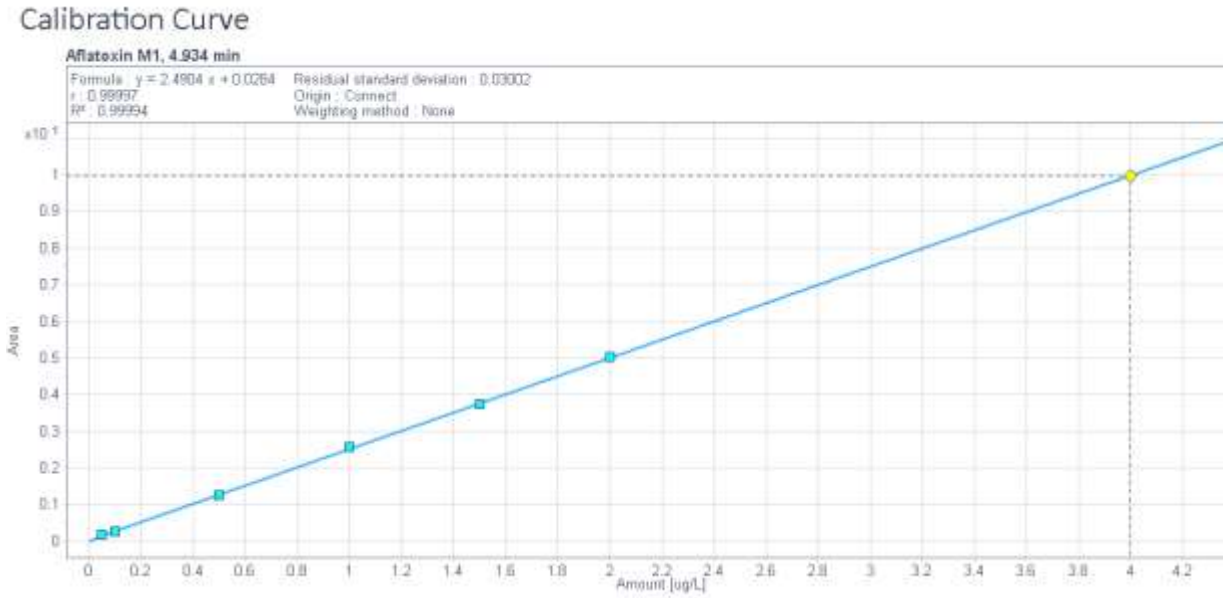
S. N	Statement	Frequency	Response	Percentage
1	Age	18-3		
		31-45		
		more than 45		
2	Gender	male		
		female		
3	Education	illiterate		
		primary		
		secondary		
		tertiary		

Part two. Assessment of KAP on mold growth and toxin formation

S.N	Attitude on mold formation and growth	Response	Frequency	Percentage
1.	Concept on mold and formation of toxin	Yes		
		No		
2	Knowledge on favorable conditions for mold growth	Yes		
		No		
3	Do you know or heard of AF	Yes		
		No		
4	Are you aware that AF cause disease in animals and humans?	Yes		
		No		
5	Do you think that milk pasteurization kill AF	Yes		
		No		
	Knowledge Farm management practice			
6	Storage place of dairy cattle feed	In house		
		In shade		
		Open field		
7	Do you regulate the feed's moisture content?	Yes		
		No		
8	Feed storage time	One week		
		Above 1 week		
9	Do you check quality of feed while buying or feeding?	Yes		
		No		

Annex 2: Calibration curve of solvent matched standards of different concentration

Figure 6. Calibration curve of solvent matched standards



Annex 3. Sample preparation, cleaning procedures

Figure 7. Photo taken during sample preparation steps

Annex 4: Laboratory Analysis

Figure 8. Photo taken during sample analysis (samples in sample vials ready for HPLC machine reading)

Annex 5. Retention time of AFM1 in different positive samples

Figure 9. Chromatographic reading of positive samples (A to C)