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ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE
DEPARTMENT OF MICROBIOLOGY, PARASITOLOGY AND POULTRY HEALTH

**DETECTION AND EVALUATION OF AFLATOXINS CONTAMINATION IN FEED
AND MILK, WITH THE ASSESSMENT OF KNOWLEDGE, ATTITUDE AND
PRACTICES OF DAIRY CATTLE OWNERS TOWARDS AFLATOXIN IN
ADDIS ABABA, ETHIOPIA**

MSc Thesis

BY

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JUNE, 2026

BISHOFTU, ETHIOPIA

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AND MILK, WITH THE ASSESSMENT OF KNOWLEDGE, ATTITUDE AND
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ADDIS ABABA, ETHIOPIA**



**A Thesis Submitted to the Department of Microbiology, Parasitology and Poultry Health,
College of Veterinary Medicine and Agriculture in Partial Fulfillment of the Requirements
for the Degree of Master of Science in One Health**

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DEDICATION

This thesis manuscript is dedicated to the beloved father, Balcha Jima Jarra, for nurturing the author with love and appreciation. His guidance and encouragements made the author succeed.

STATEMENT OF AUTHOR

I affirm that this thesis is my own original work and that all sources used in preparing it have been properly acknowledged. It has been submitted in partial fulfillment of the requirements for the MSc degree at Addis Ababa University, College of Veterinary Medicine and Agriculture, and is deposited in the University/College library to be available to borrowers in accordance with Library rules. I also affirm that this thesis has not been submitted to any other institution for the award of any academic degree, diploma, or certificate.

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LIST OF ABBREVIATIONS AND ACRONYMS

AFM1	Aflatoxin M1
AFB1	Aflatoxin B1
AOAC	Association of Official Analytical Collaboration
CAC	Codex Alimentarius Commission
ELISA	Enzyme-Linked Immunosorbent Assay
HPLC	High-Performance Liquid Chromatography
MS	Mass Spectroscopy
EAGC	Eastern Africa Grain Council
EU	European Union
FAO	Food and Agriculture Organization
HRP	Horseradish Peroxidase
IES	Institute of Ethiopian Standards
IARC	International Agency for Research on Cancer
KAPs	Knowledge Attitude and Practices
ML	Maximum Limits
NCI	National Cancer Institute
OD	Optical Density
PDI	Probable Daily Intake
UHPLC	Ultra High-Pressure Liquid Chromatography-Fluorescence Detector
US FDA	United States of America Food and Drug Authority

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ABSTRACT

Aflatoxins are toxic metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus* that contaminate foods and feeds. Aflatoxin B1 (AFB1) is a highly carcinogenic and genotoxic compound. Aflatoxin M1 (AFM1) is a hydroxylated metabolite of AFB1 formed in the liver. This study was designed to evaluate aflatoxin contamination in raw milk and feeds intended for dairy cattle, as well as to assess dairy cattle owners' knowledge, attitude and practices regarding aflatoxin and related health risks in Addis Ababa. A cross-sectional study was employed from December 2024 to May 2026, and a total of 60 pooled dairy feed and 40 raw milk samples were collected and analyzed for AFB1 and AFM1 using ELISA and Ultra-HPLC methods, respectively. The results showed that 88.3% of the feed samples were contaminated with AFB1 (276.05 ± 239.74 $\mu\text{g}/\text{kg}$), and all of these exceeded the maximum limit set by the Codex (20 $\mu\text{g}/\text{kg}$). On the other hand, AFM1 was found in only 10% of milk samples, with a mean concentration of 0.019 ± 0.01 $\mu\text{g}/\text{L}$, and all values were below the EU limit of 0.05 $\mu\text{g}/\text{L}$. A mean AFB1 concentration of 285.1 ± 250.05 $\mu\text{g}/\text{kg}$ was detected in 88.9% of pooled feeds containing commercial concentrate feed ($p = 0.0251$). In contrast, a higher AFB1 concentration of 317.7 ± 290.1 $\mu\text{g}/\text{kg}$ was detected in 90.9% of pooled feeds containing oil seed cake ($p = 0.0724$), indicating that it is an important source of AFB1. High level of AFB1 contamination was detected in 91.7% of feeds (652.2 ± 300.19 $\mu\text{g}/\text{kg}$) stored under poor conditions than feeds (87.5%) from good storage conditions (177.54 ± 54.87 $\mu\text{g}/\text{kg}$) ($p = 0.0004$). In addition, 90% of dairy cattle owners were unaware of aflatoxins in feed. Likewise, 91.8% of dairy cattle owners did not know about aflatoxin in milk. These results indicate a high level of AFB1 contamination in feed, highlighting the need for safe feed handling practices. The findings also stress the importance of increasing dairy cattle owners' awareness of aflatoxins in feed, their transfer into milk, and the possible risks to dairy cattle and public health.

Keywords: *Addis Ababa, aflatoxin B1, aflatoxin M1, dairy feed, raw milk*

1. INTRODUCTION

Aflatoxins are important contaminants in agricultural commodities such as grains and feed, and they pose serious health hazards to livestock and humans worldwide (Dhakal *et al.*, 2024). These toxic fungal secondary metabolites were first identified after an outbreak in turkeys in England, known as Turkey X disease. During this event, thousands of birds died after consuming Brazilian peanut meal (de Oliveira *et al.*, 2014). The main aflatoxin-producing fungi are *Aspergillus flavus* and *Aspergillus parasiticus*, which are common in warm, humid tropical and subtropical regions. These fungi may contaminate crops in the field, during harvest, and in storage (Grace, 2015; NCI, 2024).

Aflatoxins B1, B2, G1, and G2 are the major naturally occurring aflatoxins (David and Gary, 2024). Among them, aflatoxin B1 is the most widespread and the most strongly genotoxic and carcinogenic. Aflatoxin M1 is an important metabolite of aflatoxin B1 in animals and humans, and it may occur in milk and other dairy products from animals that have consumed feed contaminated with aflatoxin B1 (EFSA, 2021). These toxic substances can lead to acute poisoning as well as long-term outcomes such as immunosuppression and cancer development (IARC, 2012), with particularly severe effects in vulnerable groups in developing countries, including Ethiopia (Gustafson, 2018).

The Food and Agriculture Organization (FAO) estimates that aflatoxins affect about 25% of agricultural crops globally (EAGC, 2019). In many developing countries, poor storage and handling practices, together with climatic factors such as high temperatures, drought stress, and insect damage to grains, can create favorable conditions for mold growth and the resulting contamination of crops and animal feed with aflatoxins (El-Hassan *et al.*, 2022). *Aspergillus flavus* has been associated with aflatoxins contamination in many feed ingredients used for dairy cattle (Magnoli *et al.*, 2019). The intake of contaminated feed not only harms livestock health but also has serious consequences for food safety and public health (Tang *et al.*, 2023). In dairy cattle, aflatoxin contamination may cause reproductive problems, immune suppression and reduced milk production which in turn can affect food security and economic stability (Hygiene, 2024; Tang *et al.*, 2023; Jiang *et al.*, 2021).

Most government agencies across the world have set regulations on the permitted levels of aflatoxins in food for humans and animals (Hygiene, 2024). Under the European Food Ingredients Safety Code, the maximum limit (ML) for aflatoxin B1 in maize, which is mainly used as an ingredient in compound feed for dairy animals, has been set at 5 µg/kg (EFISC, 2015), while Commission Regulation (EC) 2023/915 of the European Union (EU) sets 0.05 µg/L as the limit for aflatoxin M1 in raw milk (EU, 2023). By contrast, the US FDA sets the maximum limit/action level for aflatoxin B1 in animal feed at 20 µg/kg, whereas the action level for aflatoxin M1 in milk is 0.5 µg/L (US FDA, 2011). In Ethiopia, the Institute of Ethiopian Standards (IES) has set the maximum level for aflatoxin in cow's milk and compound dairy animal feed at 0.5 µg/L and 20 µg/kg, respectively, based on international guidelines from WHO and FAO to lower risks to livestock and public health (CODEX, 2023; IES, 2019).

In Ethiopia, the extent of aflatoxin contamination in feed and its later effects on dairy production and public health have not been sufficiently studied. However, the limited available literatures reported aflatoxin contamination in animal feed, livestock milk and children's complementary food and human populations in Ethiopia (Kebede *et al.*, 2024; Zebib *et al.*, 2023; Zebib *et al.*, 2022; Gebreegziabher *et al.*, 2022; Admasu *et al.*, 2021; Gizachew *et al.*, 2016). This suggests that, although aflatoxins are recognized as a major concern for both animal and public health, there is still a clear shortage of comprehensive research on the movement of aflatoxin from dairy cattle feed (the environment) to cow's milk (the animal), and then to humans, reflecting an interconnected situation under the one health approach (Gizachew *et al.*, 2016).

This study seeks to address the gap in the detection of aflatoxins in feeds and milk, along with the related risk factors in the dairy value chain of Addis Ababa, using a one health approach. The study offer important insights into the prevalence of aflatoxin contamination in dairy cattle feed and cow's milk, as well as the knowledge, attitudes and practices of dairy cattle owners regarding aflatoxins and their public health implications. Therefore, the study aims to support the development of effective strategies to reduce aflatoxin contamination in dairy feeds and milk by examining these issues in detail.

General Objective

To detect and evaluate aflatoxin contamination in feeds and raw cow's milk from smallholder dairy cattle farms, and assess the knowledge, attitude and practices of dairy cattle owners towards aflatoxins contamination in the selected sub-cities of Addis Ababa with the one health approach.

Specific Objectives

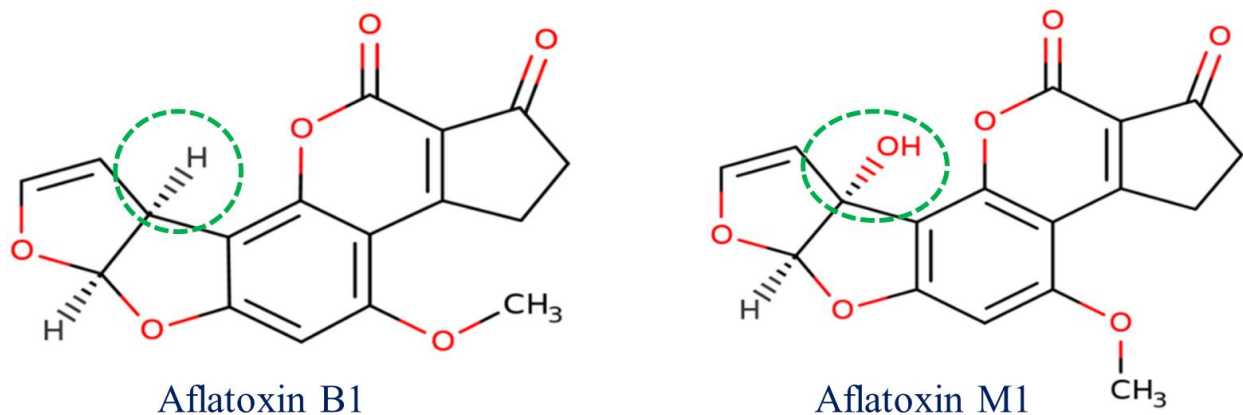
The specific objectives of the study are:

- To detect and quantify aflatoxin B1 concentrations in feeds from smallholder dairy cattle farms in Addis Ababa
- To detect and quantify aflatoxin M1 concentrations in raw cow's milk in Addis Ababa milk shed area
- To assess the knowledge, attitude and practices of dairy cattle owners towards mold growth, aflatoxin contamination and the possible health risk in Addis Ababa

2. LITERATURE REVIEW

2.1. Overview of Aflatoxins

Aflatoxins are among the most toxic mycotoxins produced by certain molds, namely *Aspergillus flavus* and *Aspergillus parasiticus*, which grow in grains, hay, decaying vegetation, and soil as secondary metabolites (WHO, 2023). These toxins were identified in the 1960s after an outbreak in turkeys in England (Turkey X disease), which led to the death of thousands of birds following the consumption of Brazilian peanut meal (Oliveira *et al.*, 2014). The main aflatoxins examined in crops are aflatoxins B1, B2, G1, and G2, classified according to their fluorescence color under ultraviolet (UV) light, with blue for B1 and B2 and green for G1 and G2 (Mostrom, 2021). In milk, however, the relevant compounds are aflatoxin M1 (AFM1) and aflatoxin M2 (AFM2), which are the metabolites of AFB1 and AFB2, respectively (Yang, 2019). Aflatoxin M1 is a hydroxylated derivative (Figure 1) formed in the liver after the intake of feed contaminated with aflatoxin B1 (Meizheng Bio-Tech, 2022).



Source: (Fermentek, 2024)

Figure 1: The molecular structures of aflatoxin B1 and a hydroxylated aflatoxin M1

Since their identification, aflatoxins have been demonstrated to be carcinogenic in laboratory animals (US FDA, 2019). Among the aflatoxins, AFB1 is the most potent mutagen, toxin, and carcinogen, and it is implicated in the development of hepatocarcinoma (CAMEO Chemicals, 2023). The IARC of the WHO classifies aflatoxins as Group 1 carcinogens, meaning they are carcinogenic to humans (IARC, 2012). AFB1 is frequently detected in contaminated grains, nuts, and seeds, especially in tropical and subtropical areas where the warm and moist conditions promote fungal growth (Mahato *et al.*, 2019). AFB2 is less toxic than AFB1, but it still presents considerable health concerns. It is commonly detected together with AFB1 in contaminated feed and food products. AFB2 is produced by *Aspergillus flavus* and is usually found in the same agricultural commodities as AFB1, including grains and oilseeds (Kumar *et al.*, 2017; Kumar, 2015).

AFG1 is generally regarded as less toxic than AFB1 being mainly produced by *Aspergillus parasiticus* and is frequently detected in contaminated agricultural commodities (Mahato *et al.*, 2019; Kumar *et al.*, 2017). AFG2 is often present together with AFG1 in contaminated food products. Similar to AFG1, AFG2 is also produced by *Aspergillus parasiticus* and is commonly detected in different crops (Mahato *et al.*, 2019). AFM1 and AFM2 are metabolites of AFB1 and AFB2, respectively. AFM1 is more often found in milk and dairy products because it may be excreted in the milk of animals that have ingested AFB1-contaminated feed. AFM1 is classified as possibly carcinogenic to humans (Group 2B carcinogen) and is a major concern in dairy products, particularly in regions where livestock are fed contaminated grains (Havelaar *et al.*, 2025; Mwakinyali *et al.*, 2018; Kumar *et al.*, 2017).

Table 1: The molecular formula and melting point of various aflatoxins

	AFB1	AFB2	AFG1	AFG2	AFM1
Molecular formula	C ₁₇ H ₁₂ O ₆	C ₁₇ H ₁₂ O ₇	C ₁₇ H ₁₄ O ₆	C ₁₇ H ₁₄ O ₇	C ₁₇ H ₁₂ O ₇
Melting point	268-269 °c	286-289 °c	244-246 °c	237-240 °c	299 °c

Source: National Center for Biotechnology Information (2026). Retrieved on May 14, 2026 from <https://pubchem.ncbi.nlm.nih.gov/compound>

Aflatoxins B2 and G2 were found to be the dihydro derivatives of the parent compounds. Though aflatoxins are relatively stable and heat-resistant compounds (Table 1) including pasteurization, they decompose when temperatures exceed their melting points (IARC, 2012).

The main producers of aflatoxins are fungal species, namely *Aspergillus flavus* and *Aspergillus parasiticus*, which are widely present in soil, decaying plant material, and on different crops, especially those rich in carbohydrates and oils, such as maize, peanuts, tree nuts, oilseeds, and spices (Kumar *et al.*, 2021; Valencia *et al.*, 2020). Conditions that support mold growth and aflatoxin formation include higher temperatures, elevated moisture and humidity, sandy soil with high organic content, crops damaged by insects, damp and poorly ventilated storage environments, and climate change, which may lead to warmer temperatures and changes in rainfall patterns (Molnar *et al.*, 2023).

Table 2: Optimal conditions for fungal growth and aflatoxin production

Fungi	Water activity (a_w) for growth	Temp. for growth	Optimal a_w for toxin production	Optimal Temp. for toxin production
<i>Aspergillus flavus</i>	0.91-0.99	15-44°C	0.99	33°C
<i>Aspergillus parasiticus</i>	0.91-0.99	15-44°C	0.99	33°C

Source: (Twinomuhwezi *et al.*, 2021)

Key: a_w: water activity of different matrices

Other environmental conditions also influence aflatoxin formation. Exposure to light lowers aflatoxin production, whereas a pH range of 5 to 7, together with easily accessible carbon and nitrogen sources, supports rapid fungal growth and increases aflatoxin synthesis (Loi *et al.*, 2023). Substrates with high carbohydrate content favor greater aflatoxin production than those rich in oils (Kumar *et al.*, 2021). Relative humidity above 85% encourages aflatoxin formation. In addition, water activity (a_w) of 0.85 or higher is ideal for aflatoxin biosynthesis, which makes drought-stressed crops more vulnerable to infection (Molnar *et al.*, 2023; Valencia *et al.*, 2020).

Changes in climate patterns, such as rising temperatures and shifts in rainfall, are likely to increase the spread of aflatoxin-producing fungi. Areas that were previously less affected may experience greater aflatoxin contamination as a result of these environmental changes (Molnar *et al.*, 2023; Valencia *et al.*, 2020). A study by Bervis and his colleagues noted that climate change-driven increases in mean temperatures are expected to alter the traditional regions where aflatoxins occur, particularly in southern Europe. A sequence of hot and dry seasons in several European countries, including Italy, Romania, Serbia and Spain, has resulted in the detection of aflatoxins in maize used for animal feed, as well as in milk from cattle fed contaminated products (Bervis *et al.*, 2021).

The presence of aflatoxins in food chains can cause major health problems, including mutagenesis, liver cancer, teratogenic effects, and immunosuppression, as well as economic losses (Balan *et al.*, 2024; Mostrom, 2021). Aflatoxins have also been linked to human health disorders such as hepatocellular carcinoma, aflatoxicosis, and chronic hepatitis. In animals exposed to aflatoxins, signs of toxicity may range from death to chronic illness, reproductive disruption, immune suppression, reduced body weight and feed intake, and lower milk yield (Hygiene, 2024; Tang *et al.*, 2023).

2.2. Mechanisms of Aflatoxin Production and Biosynthetic Pathway

The biosynthesis of aflatoxins is a complex and energy-demanding process that requires at least 27 enzymatic reactions to assemble these highly complex molecules (Loi *et al.*, 2023). This biosynthetic pathway is controlled by an intricate network of genes arranged in a biosynthetic gene cluster that serves as the precursor basis for aflatoxin compounds. Major genes involved in this pathway include aflatoxin polyketide synthase and regulatory genes such as aflR (Shabeer *et al.*, 2022).

Regulatory genes govern the expression of biosynthetic genes in response to environmental signals. Stress conditions can trigger these biosynthetic genes, resulting in higher aflatoxin production as a survival response. This biosynthetic pathway consists of a series of biochemical reactions that transform simple organic substrates into these toxic compounds. The first stage is the condensation of acetyl-CoA and malonyl-CoA by aflatoxin polyketide synthase, producing a hexaketide intermediate called 6-methylsalicylic acid. This intermediate then undergoes cyclization and additional changes, such as methylation and oxidation, which generate the bicyclic structure typical of aflatoxins. The last stages of the aflatoxin biosynthetic pathway include further enzyme-driven reactions that alter the initial polyketide product, such as hydroxylation and methylation (Shabeer *et al.*, 2022).

2.3. Aflatoxin Contamination in Animal Feed

Crops such as maize, wheat, sorghum, millet, peanut, rice, sesame, sunflower seed, and cottonseed, some of which are used as ingredients in animal feed, may be contaminated by aflatoxins (Dhakal *et al.*, 2024; Grace, 2015). A study in Mexico that assessed the presence of *Aspergillus flavus* and total aflatoxins in total mixed rations for dairy cows found a contamination level of 26.0 ± 0.4 $\mu\text{g}/\text{kg}$, with 90.4% of samples being above the locally set maximum limits (Álvarez *et al.*, 2022). Another study in Kenya reported that 86% (353/412) of feed samples collected from farmers tested positive for AFB1, and 67% (235/353) of these were above the EU limit of $5\mu\text{g}/\text{kg}$. Kang'ethe and Lang (2009) found that 81% (197/243) of feed samples from feed millers and 87% (153/175) from agrochemical shops were positive, while 58% (115/197) and 66% (92/153) of the positive samples, respectively, exceeded the EU limits (Kang'ethe & Lang, 2009).

Intensive and smallholder dairy farms are largely located in and around Addis Ababa (FAO, 2024; Gizachew *et al.*, 2016), where dairy cattle are mainly fed purchased forages, hay, wheat bran, and commercial concentrate feeds (Gebreyohanes *et al.*, 2021). A study found that 96% of dairy cattle feed samples were contaminated with aflatoxins, and 52% of these samples exceeded the European Union (EU) maximum limit of 5 µg/kg, indicating that this issue is widespread (Tang *et al.*, 2023).

Among the aflatoxins, AFB1, the most hepatocarcinogenic and hepatotoxic, may be present as a contaminant in a range of feeds (CAMEO Chemicals, 2023). Animal feed is a major route by which aflatoxins are transferred to livestock and, in turn, to humans through animal-derived products, especially milk (FAO, 2004).

Studies show that the occurrence of aflatoxins in animal feed differs markedly according to geographic setting, climate, and farming practices (Onesmo *et al.*, 2024). A study in South Korea found that AFM1 levels in raw milk were extremely low and suitable for market milk processing, which may be linked to the low AFB1 contamination in animal feed and, in turn, to the cold climate of the region (Kim *et al.*, 2010). In sub-Saharan Africa, including Ethiopia, the warm and humid climate provides favorable conditions for the growth of aflatoxin-producing fungi (Valencia *et al.*, 2020). Recent research has also shown that poor feed storage conditions on dairy farms play an important role in aflatoxin contamination, highlighting the need for urgent monitoring and control measures (Tang *et al.*, 2023; Admasu *et al.*, 2021).

2.4. Aflatoxin Contamination in Cow's Milk

The primary aflatoxin detected in cow milk is Aflatoxin M1 (AFM1), a metabolite of Aflatoxin B1 (AFB1). When dairy cows ingest feed contaminated with AFB1, part of it is converted into AFM1, which may then be secreted in milk (Min *et al.*, 2021). The movement of aflatoxins from contaminated feed into cow milk has been thoroughly documented. Studies have indicated that dairy cows are able to metabolize aflatoxins, resulting in these toxins appearing in their milk (Sassahara *et al.*, 2005). Under conditions where livestock are frequently given moldy feed, the risk of aflatoxin contamination in milk rises. In a study by Jonathan (2024), it was reported that AFB1 contamination was present in concentrate feeds, suggesting possible carry-over into milk (Jonathan *et al.*, 2024).

A study designed to assess AFM1 levels in milk from five dairy species in China found that 65.7% of cow milk, 76% of goat milk, 48% of buffalo milk, 28% of camel milk, and 18% of yak milk samples contained AFM1 above the detection limit. The same study showed that 3.1%, 9%, and 4% of the analyzed cow, goat, and buffalo milk samples, respectively, exceeded the EU maximum limit (Zheng *et al.*, 2022). Similar studies from different parts of Ethiopia have also reported variable levels of aflatoxin contamination (Table 4) in raw cow's milk and pasteurized milk which raises concerns for food safety and public health (Kebede *et al.*, 2024; Zebib *et al.*, 2023; Zebib *et al.*, 2022; Admasu *et al.*, 2021; Tadesse *et al.*, 2020; Gizachew *et al.*, 2016).

According to a study by Zentai and his colleagues, the transfer rate of AFB1 to AFM1 in milk varies substantially, with an average of 1-2% (Zentai *et al.*, 2023) and reaching as much as 6% when milk production is elevated. This conversion presents a threat to human health because AFM1 is classified as a possible human carcinogen by the International Agency for Research on Cancer (IARC, 2012).

2.5. Human Exposures to Aflatoxins and the Health Impacts on Human and Animals

Humans may be exposed to aflatoxins through the consumption of contaminated food products, including milk and dairy products from animals that have consumed contaminated feed. However, exposure most often occurs through eating contaminated plant-based foods such as peanuts, maize, rice, cereals, dried fruit, spices, and oils produced from vegetable seeds (Zentai *et al.*, 2023). Dietary exposure to aflatoxin B1 is the most common route in humans, and this toxin is one of the most potent genotoxic and carcinogenic aflatoxins, posing a threat to public health (EFSA, 2021b). Farmers and other agricultural workers may also be exposed by inhaling dust produced during the handling and processing of contaminated crops and feedstuffs (NCI, 2023).

According to a study carried out in Mali, Burkina Faso, and Niger, the estimated probable daily intake (PDI) of aflatoxins from maize was 6 to 69, 29 to 432, and 310 to 2100 ng/kg bw/day, respectively. The report also indicated that the estimated PDI of aflatoxins from another cereal crop, sorghum, examined in Mali and Niger, ranged from 2-133 and 706-2221 ng/kg bw/day, respectively (Falade *et al.*, 2022). A cohort study involving mothers and their infants in the western highlands of Guatemala found that 5% of breast milk samples and 15.7% of infant urine samples were AFM1-positive. In this study, the proportion of AFM1-positive infant urine samples was higher than that of AFM1-positive breast milk samples. This higher aflatoxin level was found in infants who had already begun consuming a mixed diet, which may have served as a route of exposure (Jolly *et al.*, 2021). Recent studies conducted in Ethiopia reported the prevalence of aflatoxin M1 contamination in human breast milk and urine, with 5.27% and 92.8% exceeding the EU maximum limit, respectively (Gebreegziabher *et al.*, 2022; Eshete *et al.*, 2021).

Exposure to aflatoxin B1 through food products, which is a common occurrence, can increase the cancer risk for humans (EFSA, 2021b). According to a research brief from the USA Feed the Future initiative, human exposure to AFM1 from milk and other dairy products is much lower than exposure to AFB1 worldwide (Havelaar *et al.*, 2025). In addition, Saha and his co-authors reported that AFM1 exposure through milk consumption does not significantly raise the global risk of liver cancer in humans (Saha *et al.*, 2022). Nevertheless, milk-derived AFM1 is classified as a human carcinogen (Marchese *et al.*, 2018).

The presence of aflatoxins in food chains can cause serious health problems, including mutagenesis, liver cancer, teratogens, immunosuppression, and economic losses (Balan *et al.*, 2024; Mostrom, 2021). Aflatoxins have also been linked to human health disorders such as hepatocellular carcinoma and aflatoxicosis, a condition marked by liver injury and other systemic effects (Shabeer *et al.*, 2022). AFB1 is known to induce acute aflatoxicosis, which may lead to liver damage, immunosuppression, and greater vulnerability to infectious diseases. Long-term exposure can result in liver cancer and other chronic health problems (Popescu *et al.*, 2022). A study reported that aflatoxins have been responsible for childhood cirrhosis in India. Cancer risk from aflatoxin exposure depends on the total dose received over a lifetime. High doses or prolonged intake of small amounts of aflatoxins in food and feed can have important health consequences for both humans and animals (Kumar, 2015). The risk is much higher in people with hepatitis B or C co-infection, since these viruses can intensify the carcinogenic action of aflatoxins (Benkerroum, 2020).

Animals that ingest sub-lethal or low concentrations of aflatoxins in their diet over several days or weeks may develop a sub-acute toxic syndrome. This condition may involve moderate to severe liver injury, reduced growth rate, lower milk or egg yield, irregular estrous cycles that are either too short or too long, and immunosuppression because of its interaction with T-cells. It may also result in lower Vitamin K activity and impaired phagocytic activity in macrophages (Dhanasekaran *et al.*, 2011). Aflatoxin can induce carcinogenesis, chronic toxicity, or acute clinical signs, depending on the species, the animal's age, the dose, and the length of exposure. Young animals of all species are more vulnerable than mature animals to the effects of aflatoxin (Dhanasekaran *et al.*, 2011). In most species, oral lethal dose (LD50) values for AFB1 range

from 0.03 to 18 mg/kg body weight. Animals exposed to aflatoxin may show toxic effects that range from death to chronic illness, reproductive disruption, immune suppression, reduced body weight and feed intake, and lower milk yield (Tang *et al.*, 2023).

2.6. Methods for Detection of Aflatoxins

The identification of aflatoxins in different matrices, including animal feed, milk, and human biological samples, is essential for food safety and public health. Therefore, the precise and rapid measurement of aflatoxin contamination is highly important (Hygiene, 2024). Representative samples collected from various locations must be sent to a laboratory equipped to test for Aflatoxin. Aflatoxins may be detected using Thin Layer Chromatography, High Performance Liquid Chromatography (HPLC), or absorbance meters such as Enzyme-linked immunosorbent assay (ELISA) readers (EAGC, 2019; Shabeer *et al.*, 2022). Of these methods, Enzyme-Linked Immunosorbent Assay (ELISA) is the most widely used, followed by High-Performance Liquid Chromatography (HPLC), Liquid Chromatography-Mass Spectroscopy (LCMS), and Thin Layer Chromatography (TLC) (Sulyok *et al.*, 2015). The more precise analytical techniques such as HPLC and MS used for aflatoxin detection are very costly, time-intensive, and demand considerable expertise (Matabaro *et al.*, 2017). Therefore, a study assessing aflatoxin testing methods showed that ELISA methods may serve as a quicker and equally dependable alternative to HPLC in routine analysis for measuring AFM1 in milk (Maggira *et al.*, 2021; Narayanan & Reddy, 2023).

ELISA is a commonly applied method for the detection and quantification of aflatoxins in feed, milk, and human samples. It is a fast and sensitive method for detecting aflatoxins, and it is suitable for screening large numbers of samples (Leszczyńska *et al.*, 2001). ELISA works on the basis of the specific interaction between antibodies and antigens. For aflatoxins, the assay usually includes coating a microplate with a specific antibody that binds aflatoxins, then adding the sample, where any aflatoxins present compete with a labeled aflatoxin, usually linked to an enzyme, for antibody binding sites. Detection is then carried out by adding a substrate that reacts with the enzyme to generate a measurable signal, usually colorimetric, which is inversely related to the aflatoxin concentration in the sample (EAGC, 2019).

2.7. The Maximum Limits of Aflatoxins Contamination in Feed and Milk

Worldwide, most government agencies have regulations on the permissible levels of aflatoxins in human and animal food. Different countries have set regulatory limits for aflatoxin concentrations in food products to protect public health. For example, the US FDA has established action levels for aflatoxins in food and feed, while the European Union applies its own strict regulations (EU, 2023; US FDA, 2019). Setting maximum limits (MLs) for contaminants in food and feed requires consideration of several factors, including toxicological evidence, analytical information (validated qualitative and quantitative data from representative samples and suitable sampling methods), intake data, technological factors, and risk assessment and risk management issues (FAO/WHO, 2023).

According to the European Food Ingredients Safety Code, the maximum limit (ML) for aflatoxin B1 in maize, which is mainly used as a compound feed ingredient for dairy animals, has been set at 5 µg/kg (EFISC, 2015). The European Union (EU) Commission Regulation (EC) 2023/915 has established a limit of 0.05 µg/kg for aflatoxin M1 in raw milk (EU, 2023). In contrast, the US FDA sets the action level for aflatoxin B1 in animal feeds at 20 ppb, while the action level for aflatoxin M1 in milk is 0.5 ppb (US FDA, 2011). Various organizations have defined action levels for aflatoxins in human food, animal feed, and animal feed ingredients, as shown in Table 3 below.

Table 3: The maximum limits of aflatoxins (AFs) in different food products and animal feed

Items	Maximum limits for AFs ($\mu\text{g}/\text{kg}$)		
	EU	USFDA	CODEX
Groundnuts (peanuts) and other oilseeds “ready to eat”	2/4*	20	4/15*
Cereals and products	2/4*	20	15*
Cereal-based food for infants and young children	-	-	5
Cereal-based food for infants and young children (for aid program)	-	-	10
Polished rice	2/5*	20	5
Dried spices	5/10*	-	4
Dairy animal feed	5	20	20
Raw milk	0.05	0.5	0.5
Corn and peanut products for cattle, swine and mature poultry	-	100	-
Complete pet food	-	20	-

Key: EU: European Union Commission Regulation; USFDA: USA-Food and Drug Administration; the symbol *: indicates that the item is destined for further processing.

These differences in the maximum permitted levels of aflatoxins indicate the varied strategies used by regulatory authorities to manage aflatoxin contamination, taking into account each country’s level of economic development, consumption limits, exposure risk to aflatoxins, dietary habits, and stringent risk assessment methods (Murokore *et al.*, 2023). The European Union (EU) Commission Regulation sets limits that are stricter than those of other regions (Table 3). However, tighter regulatory limits lead to greater food commodity waste, resulting in higher economic losses (Jean *et al.*, 2019; Manukumar *et al.*, 2017).

According to ES 6402 and CES 278 of the Institute of Ethiopian Standards (IES), the standards of maximum limits for aflatoxin levels in compound dairy feed and raw whole cow's milk are established to be 20 µg/kg and 0.5 µg/kg respectively which was adopted from the international guidelines set by the Codex Alimentarius Commission (CXS 193) of the Food and Agriculture Organization (FAO) and World Health Organization (WHO) to reduce health risks (FAO/WHO, 2023; IES, 2019).

2.8. One Health and Strategies for Mitigation and Management of Aflatoxins

One Health is a combined and integrative approach designed to maintain and improve, in a sustainable way, the health of humans, animals, and ecosystems (WHO, 2024). Food safety is among the key fields where the One Health approach is widely applied, alongside the control of zoonotic diseases and antimicrobial resistance. This approach regards human health as closely connected and mutually dependent on the health of animals and plants, as well as on the broader environment and natural ecosystems (WHO, 2022). The occurrence of aflatoxins in agricultural products and animal feed may contaminate animal-derived products such as milk and meat, creating further risks for food safety and for animal and human health (Shabeer *et al.*, 2022).

Toxin-producing fungi can contaminate crops before harvest as field fungi, harvest time, during post-harvest handling, and while in storage (FAO, 2024). Controlling aflatoxin contamination requires an integrated strategy that begins in the field before planting and continues across the whole food chain. Improved management and close monitoring of conditions before and after harvest are key measures for lowering aflatoxin contamination (Jean *et al.*, 2019). Agricultural practices such as crop rotation, the use of resistant varieties, and appropriate timing of planting and harvesting can help prevent fungal growth and the resulting aflatoxin production. After harvest, careful drying, storage, and transport are important to prevent and/or reduce aflatoxin formation and contamination (Twinomuhwezi *et al.*, 2021; Kumar *et al.*, 2021).

Various physical, chemical, and biological approaches have been used to reduce or eliminate aflatoxin contamination (Twinomuhwezi *et al.*, 2021). However, Kumar (2021) noted that these detoxification methods, including adsorbents, biological control agents, and chemical treatments, are still under investigation (Kumar *et al.*, 2021). The existing physical and chemical methods for aflatoxin mitigation and detoxification affect not only toxin levels in feed and food, but in several cases also have considerable impacts on nutritional quality and food safety. For this reason, integrated approaches should be applied to assess the toxicological and nutritional effects of these treatments (Molnar *et al.*, 2023).

The physical process of cleaning, sorting, and handpicking is considered a safe method because it does not greatly change the products (Yang, 2019). Although aflatoxins are known to be heat stable, they can be partly decomposed by high temperature treatment (237-306 °c) (Kinyoro & Kaale, 2024). In recent years, several new processing methods have been studied to achieve complete removal of aflatoxins in foodstuffs, including microwave heating, gamma and electron beam irradiation, pulsed light, electrolyzed water, and cold plasma (Yang, 2019). One study found that ultraviolet rays at a wavelength of 278 nm, with 100% irradiation energy for 10 min, reduced aflatoxins by more than 80%. Treating food products, including animal products, with a γ -ray source is moderately effective, with an average reduction of 65% at a high irradiation dose (Yang, 2019).

Studies on the chemical detoxification of aflatoxins have indicated that a one-hour ammonia treatment can reduce aflatoxin B1 levels effectively and permanently. Treatment with NH₄OH at high temperature or with gaseous NH₃ can also markedly lower aflatoxin B1 content, sometimes by more than 99% (Yang, 2019). In a study examining the effect of hydrochloric acid on AFB1 degradation in aflatoxin-contaminated corn gluten, AFB1 breakdown increased as the HCl concentration rose, and complete degradation occurred in the presence of 5 mol/L HCl after 4 hours at 110 °c (Aly & Hathout, 2011). Another study found that 89.4% of AFB1 in peanuts was decomposed by ozone at a concentration of 50 mg/L and a flow rate of 5 L/min for 60 hours (Diao *et al.*, 2013).

Among bacteria, *Bacillus*, *Pseudomonas*, *Lactobacillus*, and *Streptomyces* are the main genera reported to inhibit *Aspergillus* spp. by restricting mycelial growth, suppressing conidial germination, and reducing aflatoxin production through competition and antibiosis (Ren *et al.*, 2020). *Flavobacterium aurantiacum* NRRL B-184, a bacterium isolated from soil and water, was found to detoxify or irreversibly remove aflatoxin from contaminated milk, oil, peanut butter, peanuts, and corn, and to remove it partially from soybeans (Ciegler *et al.*, 1966). In another study, *L. plantarum* reduced toxin levels by 89% when AFB1-contaminated milk was exposed to the bacterium at 37 °c for different time periods. The aflatoxin was bound to the bacterial cell wall under optimal temperature and pH conditions (Kinyoro & Kaale, 2024).

Atoxigenic fungal strains such as *Aspergillus niger*, *Eurotium herbariorum*, *Rhizopus* sp., and non-aflatoxin-producing *Aspergillus flavus* were able to convert AFB1 to aflatoxicol-A (AFL-A), which could then be converted to aflatoxicol-B (AFL-B) through the action of medium components or organic acids produced by the fungi. The fungus *Penicillium raistrickii* NRRL 2038 was able to convert AFB1 into a new compound similar to AFB2 (Yang, 2019). *Rhizopus oligosporus* was able to inhibit AFB1 synthesis or degrade AFB1 when cultured together with the AFB1-producing fungus *A. flavus* (Kusumaningtyas *et al.*, 2006).

A broad variety of inhibitory compounds has been reported, including enzymes such as chitinases, proteases, and glucanases, as well as peptides, organic acids like lactic and fatty acids, and other alicyclic and aromatic compounds (Ren *et al.*, 2020). Certain enzymes capable of degrading aflatoxins have also been isolated and purified from microbial sources. This new enzyme can break the lactone ring of aflatoxin. Enzymatic degradation of aflatoxins has several advantages including avoiding changes in flavor and preserving nutritional value (Yang, 2019).

Chlorophyllin, as a sorbent added to contaminated feeds, may lower AFB1-DNA adduct formation (Yang, 2019). Clay acts in a manner similar to chlorophyll and chlorophyllin. When clay is incorporated into animal feeds, AFM1 levels in milk decrease in line with reduced AFB1 absorption (Phillips *et al.*, 2008; Jean *et al.*, 2019). A study evaluating the capacity of low-cost, locally available adsorbents, including activated charcoal, bentonite, and fuller's earth, to detoxify poultry feed contaminated with aflatoxin found that bentonite was the most effective adsorbent, reducing the total aflatoxin concentration from $120 \pm 38 \mu\text{g/kg}$ to $15 \pm 5.0 \mu\text{g/kg}$ (Mgbeahuruike *et al.*, 2018).

2.9. Current Status of Aflatoxin Contamination in Ethiopia

Ethiopia's warm and humid climate creates suitable conditions for the development of molds such as *Aspergillus flavus* and *Aspergillus parasiticus*, which grow well in these environments and are known aflatoxin producers (Gelaye, 2024). In a systematic review by Gelaye (2024), maize samples in Ethiopia showed a high average aflatoxin level of $864.66 \mu\text{g/kg}$ greatly exceeding the international safety limit of 20 ppb. In addition, groundnuts showed substantial contamination, with levels reaching $61 \mu\text{g/kg}$, while spices and animal feeds often exceeded the maximum allowable limits (Gelaye, 2024).

Existing studies in Ethiopia (Table 4) show the presence of aflatoxin contamination in animal feed (Gizachew *et al.*, 2016), livestock milk (Zebib *et al.*, 2022; Zebib *et al.*, 2023; Kebede *et al.*, 2024; Admasu *et al.*, 2021), children's complementary food, and human populations in Ethiopia (Gebreegziabher *et al.*, 2022). This suggests that, although aflatoxins are increasingly recognized as a major public health issue, there is still a clear shortage of comprehensive research on the linked conditions of their origin and transfer into milk within a one health approach (Gizachew *et al.*, 2016).

In Ethiopia, aflatoxin contamination of food products is worsened by a number of factors, such as poor agricultural practices, climatic conditions, and limited awareness. Insufficient drying, improper harvesting, and poor storage conditions promote the growth of fungi that produce aflatoxins. Traditional storage methods often do not shield crops from moisture and pests, which increases fungal growth (Gelaye, 2024). Many farmers are unaware of proper agricultural practices and the dangers linked to aflatoxin contamination, and this helps sustain the problem (Degefe & Geleta, 2024).

Table 4: Studies conducted on aflatoxins contamination in animal feeds, cow’s milk, and human samples in Ethiopia

S. N	Study Area	Sample type		Total sample	Positive	AF type	AF Contamination level (µg/kg);(µg/L)		Sample ≥ EU ML	Technique	References
							Median	Mean			
1	S/Gonder Zone	Feed	Hay	100	96	AFT	22.03	19.0	7	ELISA	Tang <i>et al.</i> , 2023
			Pasture	100	-	„	65.59	27.89	11	„	„
			Concentrates	100	-	„	112.88	88.0	23	„	„
2	AA Milk shed	Feed, Milk	Feed	156	156	AFB1	18	97	156	ELISA	Gizachew <i>et al.</i> , 2016
			Raw milk	110	102	AFM1	0.094	0.41	29	„	„
3	Sululta & Sabata	Cow milk	Raw milk	60	30	AFM1	-	0.047	10	HPLC	Kebede <i>et al.</i> , 2024
			Pasteurized milk	54	52	AFM1	-	0.046	19	„	„
4	Oro, Am	Cow milk	Raw milk	64	-	AFM1	0.08	0.32	-	ELISA	Zebib <i>et al.</i> , 2023
5	Oro, Am SNNP		Raw milk	64	64	AFM1	0.084	0.319	40/14*	„	Zebib <i>et al.</i> , 2022
			Pasteurized milk	64	64	AFM1	0.101	0.324	43/16*	„	„
6	S/Gondr		Raw milk	100	99	AFM1	0.98	0.47	41	„	Admasu <i>et al.</i> , 2021
7	Bishoftu		Raw milk	52	52	AFM1	-	0.690	104/	„	Tadesse <i>et al.</i> , 2020
		Pasteurized milk	56	56	AFM1	-	0.970	89*	„	„	
8	Sidama	Human	Breast milk	360	232	AFM1	0.0011	-	19	„	Eshete <i>et al.</i> , 2021
9	Shebe S/E		Human Urine	408	379	AFM1	5.27	-	379**	„	Gebreegiabher <i>et al.</i> , 2022

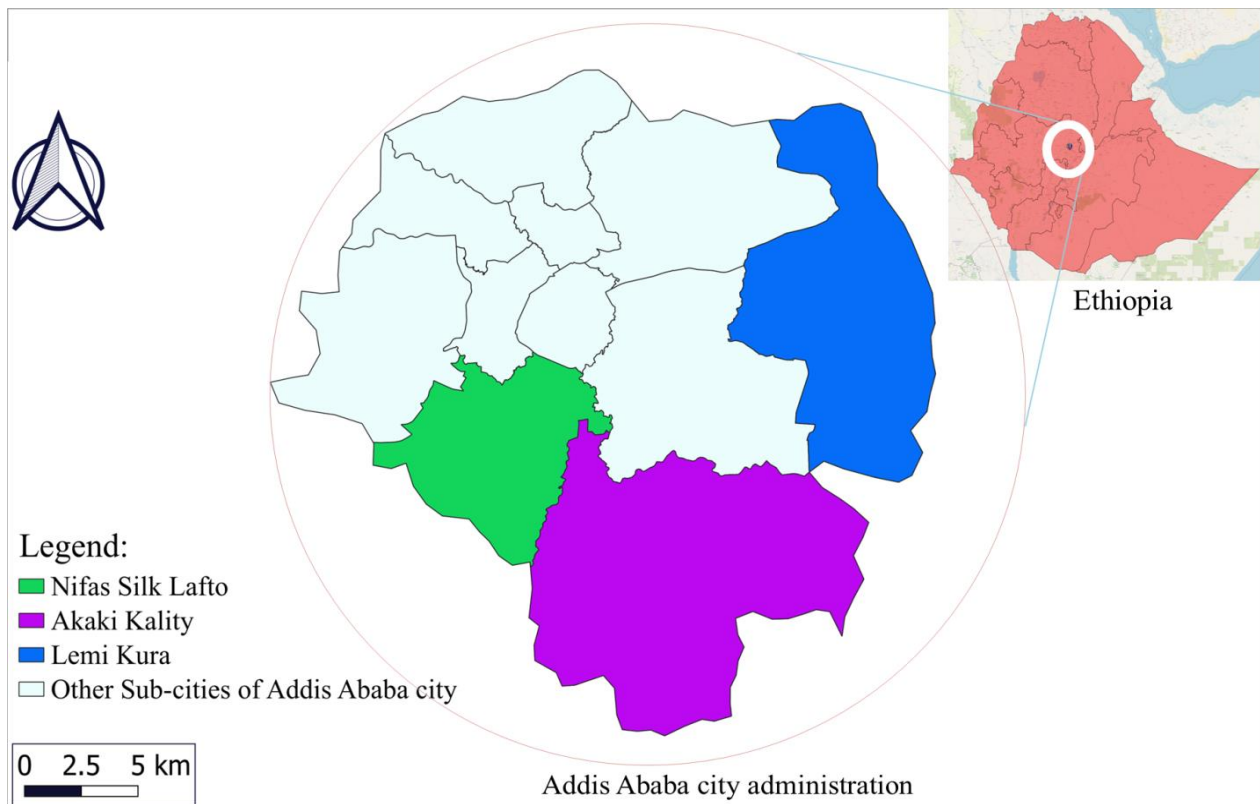
Key: AFT: Total aflatoxins, AFB1: Aflatoxin B1; AFM1: Aflatoxin M1; EU ML: European Union maximum limit for Dairy feed = 5 µg/kg / [20ppb]*; Human breast milk = 0.25 µg/L; Cow milk = 0.05 µg/L / [0.5ppb]*. The symbol * indicates CODEX/ Institute of Ethiopian standards maximum limit.

Compared with the relatively large number of studies on aflatoxins in various animal feeds, as well as raw and pasteurized milk, only a few studies have examined the presence of aflatoxin in humans (Table 4). A study in the Sidama region of southern Ethiopia found detectable AFM1 in 64.4% of 360 breast milk samples, and 5.3% (19/232) of these samples were above the 0.025 ppb threshold established by the European Union for infant milk (Eshete *et al.*, 2021). In another study, urinary AFM1 (AFM1/creat) was identified in 93% (379/408) of school-age children in southern Ethiopia, with a median level of 480 pg/mg (Gebreegziabher *et al.*, 2022).

3. MATERIAL AND METHODS

3.1. Study Area and Period

The study was carried out in selected sub-cities of Addis Ababa, Ethiopia, from November 2024 to May 2026. Addis Ababa lies at an altitude of about 2,355 meters above sea level. The area records a minimum annual temperature of 7 °c and a maximum annual mean temperature of 23 °c (Climate, 2023). The highest temperatures occur during the main dry season (January to May), while the lowest are experienced in the wet season (June to August). The city receives an annual rainfall of 1165 mm. It also has relatively high humidity, with an annual average of 64.1%, and monthly averages ranging from 51% in February to 85% in August (Weather, 2024).



Map was developed using QGIS Desktop software version 3.40.10 (<https://qgis.org/>)

Figure 2: Map showing study area location

Addis Ababa is divided into eleven sub-cities (figure 1), of which Akaki Kaliti, Nefas Silk Lafto, and Lemi Kura were selected for the present study. These sub-cities are recognized for a relatively higher dairy cattle production aimed at satisfying the demand for milk in Addis Ababa city. The main feed inputs for dairy farms in the city include agro-industrial by-products such as wheat bran and oilseed cakes, along with purchased roughage and commercial concentrate feed (FAO, 2024). In the smallholder dairy cattle farms visited during the study period, packages of different feed types were kept on raised wooden boards in the store, while some farms stored the purchased feeds in dry containers.

3.2. Study Design and Sample Size Determination

A cross-sectional study design was used to collect pooled feed samples and raw cow's milk samples from the selected smallholder dairy farms in the chosen sub-cities. The sample size was calculated using a statistical formula for proportion (Thrusfield, 2007):

$$n = (Z^2 \times p(1-p)) / d^2$$

Where: n = required sample size; Z = Z -value (1.96 for 95% confidence level); p = expected/estimated prevalence of aflatoxin contamination; D = margin of error (precision) (5%)

Assuming a 96% prevalence of aflatoxin B1 in dairy feed from a previous study (Tang *et al.*, 2023) that employed the same detection method (ELISA technique), and using a 5% margin of error in the formula above, 60 dairy feed samples were calculated to be collected. In contrast, a 99% expected prevalence of aflatoxin M1 (Admasu *et al.*, 2021) from another study was used to estimate the required number of raw cow's milk samples, giving a sample size of 15. However, the raw cow's milk sample size was increased to 60 to match the number of feed samples, improve precision, increase the reliability of the findings, and account for the possible loss of samples. Nevertheless, because of limited reagents such as immunoaffinity columns, only 40 raw cow's milk samples were analyzed. All of the dairy farm owners (60) from which the feeds and milk samples were collected were the participants of the questionnaire survey.

3.3. Study Population

The study population comprised intensive smallholder dairy farms in different sub-cities of the Addis Ababa city administration, from which feed and milk samples were collected for analysis. Adult owners of these dairy farms from which the feed and milk samples were obtained the study population for the questionnaire survey.

3.4. Sampling Method

Three sub-cities, namely Akaki Kaliti, Lemi Kura, and Nefas Silk Lafto, were purposively selected because they had a relatively larger number of intensive smallholder dairy farms, and their accessibility was also considered. The list of dairy farms in the study area was obtained from the wereda urban agriculture officers and used as the sampling frame. Simple random sampling, using a lottery method, was then applied to select the dairy farms. Representative pooled dairy feed samples were collected from the currently used feed stores of the farms. All types of dairy cattle feed per each farm available on the day of sample collection were combined into pooled samples. Corresponding pooled cow's milk samples were also collected from the same dairy farms. Adult dairy cattle owners from the selected farms were invited to take part in the questionnaire survey.

3.5. Data Collection Methods

Following the collection of feed and milk samples, laboratory tests were carried out to determine whether aflatoxins were present and to measure their concentrations. The findings were entered on a paper-based form prepared to record all relevant details for aflatoxin B1 and M1 analysis. In addition, a questionnaire survey/interview was used to gather information on people's knowledge, attitudes, and practices regarding mold growth and aflatoxin contamination in dairy feeds and raw cow's milk, as well as the feed handling conditions on dairy farms.

3.5.1. Feed sample collection and preparation

Pooled dairy feed samples were obtained from intensive smallholder dairy farms in the selected sub-cities of Addis Ababa. After the feed contents in each carrier bag or container were thoroughly mixed, a 500 g pooled feed sample was taken from each dairy farm store.

3.5.2. Laboratory analysis of feed with ELISA method

The feed samples were labeled, transported to the laboratory, and dried in a hot air oven at 50 °c for 48 hrs. The dried pooled feed samples were then thoroughly mixed and ground. The extraction solvent (80% methanol; 20% distilled water) was prepared by adding 40 ml of distilled water to 160 ml of methanol for each sample. A 200 ml portion of 80% methanol was poured into a 500 ml screw-cap glass bottle, after which 2 g of the ground feed sample was added and mixed by shaking for 10 minutes at room temperature. The sample-to-solvent ratio was 1:100 dilution (w/v). The mixture was then filtered through Whatman filter paper to remove solid particles, and the filtrate was collected for analysis.

The Phosphate Buffered Saline (PBS)-Tween packet supplied with the kit (Helica Afla B1 Low Matrix ELISA kit) was prepared by rinsing its contents into a 1-liter container with a gentle flow of distilled water until the final volume reached 1 liter. This solution was then used as the sample dilution buffer and wash buffer in the assay procedure. A 100 µl portion of the filtrate or extract aliquot was mixed with 900 µl of the sample dilution buffer in a small sample tube, giving a filtrate-to-dilution buffer ratio of 1:10. Consequently, the final dilution used for calculation was 1:1000.

The final diluted samples were examined for AFB1 contamination levels using a direct competitive ELISA method with a Helica Afla B1 Low Matrix ELISA kit (Aflatoxin B1 Low Matrix ELISA Kit (Cat No. 981BAFL01LM-96) (KIT5005)). Before the assay began, all reagents in the Helica Afla B1 LM ELISA kit were allowed to reach room temperature (Hygiene, 2024). The mixing well was set in a microwell holder for each standard and each sample to be tested in duplicate, and 200µl of assay diluent was added to each mixing well. Using a fresh pipette tip each time, 100µl of each standard (0.0, 0.02, 0.05, 0.1, 0.2 and 0.4 ng/mL) and the prepared sample was transferred to the mixing well containing diluent and mixed by priming the pipettor 3 times.

Using a fresh pipette tip for each sample, 100 µl from every mixing well was transferred into the matching antibody-coated microtiter well and left at room temperature (37°C) for 30 minutes. The contents of each microwell were then removed with an automated microwell washer, and the wells were washed by filling them with PBS-Tween wash buffer. The wash solution was discarded, and this washing step was repeated three times to eliminate unbound material. After that, 100 µl of Aflatoxin horseradish peroxidase (HRP)-conjugate was added to each antibody-coated well and incubated at room temperature for 30 minutes while covered to protect it from direct light. The microwells were again washed in the same manner using the automated washer. A substrate solution (100 µl) was added to each microwell and left at room temperature for 10 minutes. After this, 100 µl of stop solution was added in the same order and at the same rate as the substrate solution.

Finally, the optical density (OD) of each microwell was measured with a microtiter plate reader (spectrophotometer) at 450 nm. The duplicate readings for each standard and feed sample were averaged. The data analysis worksheet for Aflatoxin B1 Low Matrix ELISA Kit (Cat No. 981BAFL01LM-96) (KIT5005) was used to determine the final Aflatoxin B1 concentration in each feed sample by using the equation: $\%B/B_0 = (OD_{\text{sample}}/OD_{\text{zero standard}}) * 100$. The calibration curve was drawn from the responses of the standard solutions of AFB1 where the $r^2 = 9897$. The calibration curve method of statistical approach was used to calculate the limit of detection (LOD) and limit of quantification (LOQ) of the test method (*i.e.* $LOD = (3.3 * SD)/S$ whereas, $LOQ = (10 * SD)/S$). *Where:* SD = Standard deviation (the residual standard error of the linear regression line); S = Slope of the calibration curve.

3.5.3. Milk sample collection and preparation

A pooled raw cow's milk sample of approximately 100 ml was collected from each selected dairy farm and labeled with the relevant information. The samples were then transported to the laboratory in cool boxes and kept at -20°C until analysis. Sample preparation was carried out according to the guideline of the Association of Official Analytical Chemists 2000.08 (AOAC, 2000).

3.5.4. Laboratory analysis of milk with Ultra-HPLC system

The frozen raw cow's milk sample was thawed in a water bath at a temperature of 37°C. The milk sample was centrifuged at 4000 g for 10 minutes at 10°C to separate the fat. The upper fatty layer was discarded carefully by using spatula and filtered through Whatman No. 4 filter paper to remove any particulate matter.

After cleaning the immunoaffinity column with a 10 ml of distilled water at a rate of 3 drops/second, the filtered milk sample was applied/ loaded to the immunoaffinity column at a rate of 1 drop/second. The column was rinsed with 10 ml of distilled water and allowed to air dry. Next, the AFM1 was eluted from the column using 4 ml of pure acetonitrile and collected in a falcon tube to be evaporated under nitrogen evaporater at a temperature of 40°C to reduce the volume and increase the concentration of AFM1 for detection (ISO, 2021). The final residue was reconstituted with 200 µl of a mobile phase solution (65% distilled water, 25% acetonitrile, and 10% methanol) and transferred to small amber vials making it ready for detection.

A mobile phase solution was pumped into the Ultra-HPLC system at a steady flow rate till a stable baseline was developed. All AFM1 standard solutions and samples were placed in the storage rack of the Ultra-HPLC so that 50 µl of the concentrated eluent was automatically drawn out and injected into the main Ultra-HPLC flow path which led to the C18 reversed-phase analytical column for separation of AFM1 band. Then, detection of AFM1 was done with fluorescence detector which flashes a UV light beam at an excitation wavelength of 365 nm and an emission wavelength of 435 nm.

The area under the peak detector responses from the chromatogram matching the retention time (11.98 minutes) of the standard concentration were considered in computing the mass concentration of AFM1. The calibration curve for AFM1 standard solutions with concentrations of 0.25, 0.50, 1.00, 2.00, 4.00, 8.00, and 10.0 µg/L was constructed to test the validation of the test method. The linearity which was indicated by the coefficient of determination ($r^2 = 0.997$) was determined through linear regression analysis of the calibration curve. Aflatoxin M1 mass concentration (µg/L) of the raw milk samples were calculated on a worksheet designed for the determination of Aflatoxin M1 in Milk by using the equation below:

$$W_m = W_a \times (V_f/V_i) \times (1/V_s)$$

Where: W_m : the mass concentration of AFM1 in the original test sample ($\mu\text{g/L}$); W_a : the amount of AFM1 corresponding to area or height of the AFM1 peak of the sample extract (ng); V_f : the final volume of the redissolved eluate (μL); V_i : the volume of the injected eluate (μL); V_s : the volume of prepared test portion passing through the immunoaffinity column (mL) (George, 2019).

3.5.5. *Assessment of Knowledge, attitude and practices*

A questionnaire survey was implemented through a face to face interview to understand the knowledge, attitude and practices of dairy cattle owners regarding to mold growth and aflatoxins contamination in dairy feeds and human foods, aflatoxin contamination in raw cow's milk, dairy feed handling and storage, and the possible health risk of aflatoxins on dairy cows and humans. The knowledge level of the respondents was determined after calculating their raw knowledge test score based on Bloom's Cut-off Point (Bloom *et al.*, 1956).

3.6. **Data Analysis**

Data were entered into Microsoft Office Excel 2010, then exported to Stata version 14 (Stata 421.14.0.372) for analysis. Descriptive statistics were applied to summarize the dataset, including the mean, standard deviation, maximum, and minimum values. A t-test was then used to examine hypotheses concerning aflatoxin contamination and the related risk factors. Pearson χ^2 test was used to assess the association between the knowledge level of dairy cattle owners and the risk factors/variables.

3.7. Ethical Considerations

Ethical clearance for the study was obtained from the institutional animal research ethics review committee of the College of Veterinary Medicine and Agriculture, Addis Ababa University (Ref. No.: VM/ERC/04/119/17/2026), and the Aklilu Lemma Institute of Health Research (AL-IHR), Addis Ababa University (Ref. No.: ALIPB IRERC/174/2017/25). Study participants were enrolled only after they voluntarily agreed to take part. All information collected from the dairy producers, the brand names of the commercial dairy feeds, and the individuals involved in the study will remain confidential and will not be disclosed.

4. RESULTS

In the present study, a total of 60 dairy cattle farms were visited in the Akaki Kality (34), Nifas Silk Lafto (16), and Lemi Kura (10) sub-cities of Addis Ababa city. Pooled dairy cattle feed samples were collected from each farm. However, raw cow's milk samples were obtained only from 40 farms. The 60 dairy cattle owners were interviewed on the same day as the farm visit to assess their knowledge, attitudes and practices.

4.1. Aflatoxin B1 contamination in dairy feeds

Of the pooled feed samples obtained from smallholder dairy farms, 88.3% (53/60) were contaminated with aflatoxin B1 (AFB1). Half of the feed samples had AFB1 concentrations below the median value of 201.11 $\mu\text{g}/\text{kg}$ with the IQR of 89.55 $\mu\text{g}/\text{kg}$. The mean AFB1 contamination level was 276.05 $\mu\text{g}/\text{kg}$, with a standard deviation of 239.74 $\mu\text{g}/\text{kg}$. The limit of detection (LOD) of the test method for AFB1 was 1.022 $\mu\text{g}/\text{kg}$. Most of the AFB1-positive pooled dairy feed samples (62.26%) came from Akaki Kality sub-city, as presented in Table 5.

Table 5: AFB1 levels detected in pooled dairy feeds from selected sub-cities of Addis Ababa by using ELISA method

Sub-cities	Samples (n)	Positive (%)	> 20 $\mu\text{g}/\text{kg}$ (%)	Mean \pm SD	Range
Akaki Kality	34	33 (97.05)	100	305.25 \pm 270.05	23.17-1021.25
Nifas-silk Lafto	16	11 (68.7)	100	258.94 \pm 232.95	67.18-944.24
Lemi Kura	10	9 (90)	100	189.92 \pm 42.25	122.24-243.09
Total	60	53 (88.3)	100	276.05\pm239.74	23.17-1021.25

The level of AFB1 contamination in the pooled dairy feed samples was above the maximum permissible limit of 20 µg/kg established by the Codex Alimentarius Commission of the FAO/WHO and the Institute of Ethiopian Standards (ES 6403).

All smallholder dairy farms included in the present study used hay as a common feed type. Other feed types frequently used on these farms were wheat bran/furushka (95%), commercial concentrate feed (90%), industry by-products such as oil seed cake (55%), and pea hull (43.3%). As shown in Table 6 below, pooled feeds contaminated with AFB1 and containing commercial concentrate feed and oil seed cake differed significantly ($p \leq 0.05$). The test statistic (t-test) indicated that the inclusion of pea hull in the mixed ration was not associated with a statistically significant difference ($p = 0.9628$).

Table 6: AFB1 contamination level in pooled dairy feeds and the associated risk factors

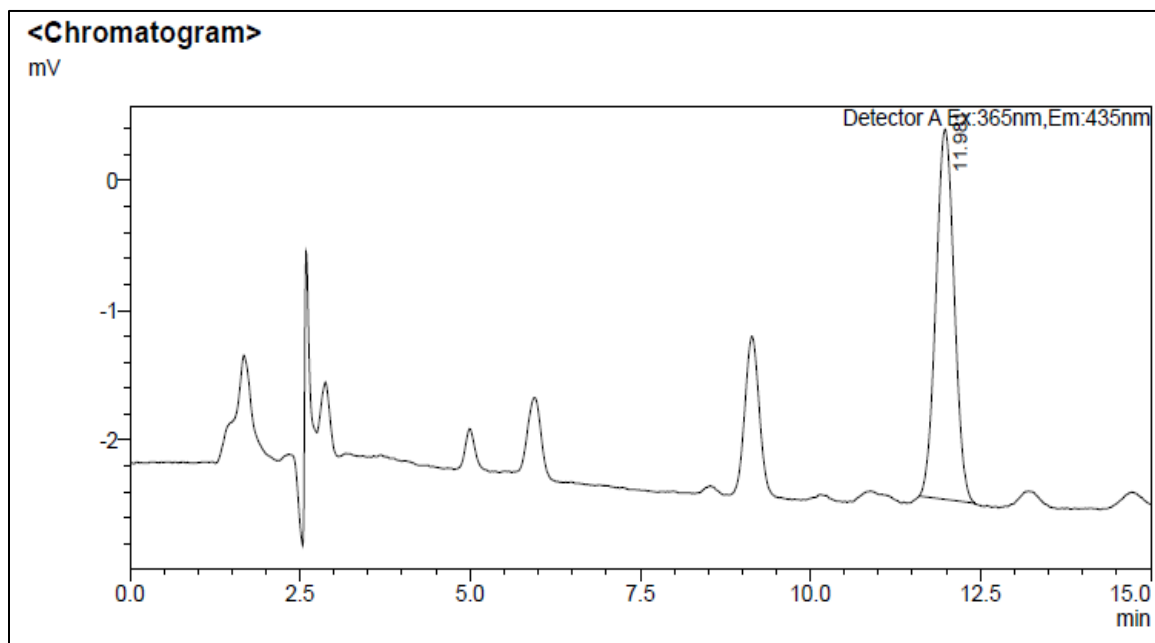
Variables	AFB1 contamination				
	Samples (n)	Positive (n)	%	Mean \pm SD	P-value
With commercial concentrate feed	54	48	88.9	285.1 \pm 250.05	
Without commercial concentrate feed	6	5	83.3	188.9 \pm 44.8	0.0251
With Oil seed cake	33	30	90.9	317.7 \pm 290.1	
Without Oil seed cake	28	24	85.7	225.6 \pm 150.2	0.0724
With Pea hull	26	24	92.3	268.0 \pm 243.9	
Without pea hull	34	29	85.3	282.7 \pm 240.2	0.8267
Feed storage conditions					
Good	48	42	87.5	177.54 \pm 54.87	
Poor/ Needs improvement	12	11	91.7	652.2 \pm 300.19	0.0004

Key: Feed storage conditions judged based on the assessment by observation. Good = if feed placing area is dry, well ventilated store and have feed placing wooden board raised up from the floor; Poor = if one/more of these conditions are lacking

The feed storage conditions in most of the dairy farms (80%) were good, characterized by dry floors and walls, a well-ventilated store, and a raised wooden board for placing feed. In contrast, 20% of the visited dairy farms had poor feed storage conditions. The results showed that feed storage conditions in the farms have statistically significant difference ($p = 0.0004$).

4.2. Aflatoxin M1 contamination in raw cow's milk

The analysis report achieved from the UHPLC system after fluorescence detection of AFM1 at the specific retention time (Ret. Time) can be shown in the Figure 3 below.



Key: Ex: Excitation; Em: Emission; min: minutes; nm: nanometers; millivolts

Figure 3: A representative UHPLC analysis report of 0.25 ppb standard concentration

Out of the 40 raw cow's milk samples examined, only 4 (10%) contained aflatoxin M1 (AFM1) contamination. The average AFM1 concentration was 0.019 $\mu\text{g/L}$, and the highest value recorded was 0.03 $\mu\text{g/L}$, as shown in Table 7.

Table 7: AFM1 levels detected in raw cow’s milk from selected sub-cities of Addis Ababa by using Ultra-HPLC method

Sub-cities	Samples (n)	Positive	Exceeding 0.05 µg/L (%)	Mean ±SD	Range
Akaki Kality	26	4 (15.3%)	0	0.019±0.01	0.009-0.03
Nifas-silk Lafto	7	0	-	-	-
Lemi Kura	7	0	-	-	-
Total	40	4 (10%)	0	0.019±0.01	0.009-0.03

None of these AFM1-contaminated milk samples exceeded the stricter maximum permissible limit set by the EU (0.05 µg/L) or the Institute of Ethiopian Standards CES 278 (0.5 µg/L).

4.3. Correlation of AFB1 positive feed samples and AFM1 positive milk samples

The relationship between the AFM1-contaminated (10%) raw cow’s milk samples identified by Ultra-HPLC and the matching AFB1-contaminated pooled dairy feed samples detected by ELISA is presented in Table 8 below.

Table 8: The levels of AFB1 (ELISA) in dairy feeds and levels of AFM1 (UHPLC) in raw milk

Sample ID	Aflatoxin B1 in feed (µg/kg)	Aflatoxin M1 in milk (µg/L)
1	96.18	0.01
2	1021.25	0.03
4	655.10	0.02
16	375.42	0.01

All aflatoxin M1 positive raw milk samples came from farms with aflatoxin B1 positive feed samples.

4.4. Knowledge, attitude and practices of smallholder dairy cattle owners

Socio-demographic characteristic of dairy cattle owners is shown on table 9. Of all smallholder dairy cattle owners who took part in this questionnaire survey, 85% (51/60) were male. The respondents' dairy farm work experience ranged from 1 year to 41 years. The average length of dairy farm work experience was 14.1 years, with a standard deviation of 10.71 years. Most respondents (70%) had been involved in dairy farming for more than 6 years.

Table 9: Socio-demographic characteristic of dairy cattle owners

Variables	Frequency (%)
Gender	
Male	51 (85)
Female	9 (15)
Education level	
No formal education	2 (3.3)
Primary (1-8)	25 (41.7)
Secondary (9-12)	21 (35)
Tertiary	12 (20)
Experience in dairy farming activity	
≤ 5 years	18 (30)
6-15 years	19 (31.6)
≥16 years	23 (38.3)

Majority of the respondents (80%) have at least primary education. All respondents (60/60) were aware of mold growth on dairy cattle feeds and human foodstuffs. However, only a small proportion knew that dairy feeds (10%) and human foodstuffs (1.67%) can be contaminated by aflatoxins. All participants (60/60) in the current study stated that poor feed storage conditions, such as wet floors, insufficient ventilation, and feeds placed directly on the floor, can promote mold growth in dairy feeds.

On the other hand, 41.7% (25/60) of the respondents indicated that using contaminated or moldy agricultural and industrial by-products as feed ingredients can also lead to mold growth in dairy cattle feeds. The responses of the study participants indicated that moisture (60/60), warm temperature (59/60), poor ventilation (60/60), and prolonged feed storage (60/60) can create conditions that favor mold growth.

According to the participants' knowledge assessment, only 8.33% (5/60) of the respondents were aware of aflatoxin contamination in raw cow's milk. Most of these respondents (4/5) believed that the cause or source of aflatoxin contamination in milk is the feeding of dairy cattle with aflatoxin-contaminated feed. Table 10 below presents a summary of the knowledge and practices of smallholder dairy farm owners who took part in the current study.

Table 10: Knowledge, attitude and practices of dairy cattle owners towards mold growth and aflatoxins contamination

Dairy cattle owners' knowledge	Respondents (n)	Frequency (%)
Know about mold growth in dairy feeds	60	60 (100)
Know about mold growth in foodstuff	60	60 (100)
Know about aflatoxins contamination in feeds	60	6 (10)
Know about aflatoxins contamination in foodstuff	60	1 (1.67)
Know the favorable conditions for mold growth in dairy feeds	60	60 (100)
Know about aflatoxin contamination in raw cow's milk	60	5 (8.33)
Know that contaminated dairy feed is the cause/ source for aflatoxin contamination in raw cow's milk	5	4 (80)
Dairy cattle owners' attitude		
Think that mold may cause health impact on humans	60	59 (98.33)
Think that mold may cause health impacts on dairy cows	60	59 (98.33)
Think that aflatoxins may cause disease in dairy cows	6	5 (83.33)
Think that aflatoxins may cause disease in humans	5	5 (100)
Dairy cattle owners' practices		
Store feeds on a dry area	60	58 (96.7)
Have a well-ventilated feeds store	60	40 (66.66)
Store feeds for a short period of time (≤ 1 month)	60	56 (93.33)
Avoid a feed if mold is grown on it	60	58 (96.7)
Don't use a foodstuff intended for humans consumption if mold is grown on it	60	60 (100)

Among the small number of respondents (5/60) who demonstrated good knowledge of aflatoxin contamination in cow's milk, 80% (4/5) identified the cause or source of the contamination. The respondents (80% (4/5)) who recognized aflatoxin contamination in feed and cow's milk, as well as its causes, believed that aflatoxin could pose health risks to dairy cattle and humans. The findings of the present study showed that the overall knowledge of 56.6% (34/60) of dairy cattle owners towards of mold growth and aflatoxin contamination in feeds, foods, and cow's milk is low (Table 11). The remaining 36.6% (22/60) and 6.6% (4/60) of the respondents had moderate and high levels of overall knowledge, respectively.

Table 11: Knowledge level of dairy cattle owners associated with education level and training status

Education level	Respondents	Knowledge level			P-value
		High	Medium	Low	
No formal education	2 (3.3%)	0	0	2 (100%)	0.054
Primary (1-8)	25 (41.6%)	1 (4%)	5 (20%)	19 (76%)	
Secondary (9-12)	21 (35%)	1 (4.7%)	10 (47.6%)	10 (47.6%)	
Tertiary	12 (20%)	2 (16.6%)	7 (58.3%)	3 (25%)	
Total	60	4 (6.6%)	22 (36.6%)	34 (56.5%)	
Received training on feed management					
Yes	29 (48.3%)	3 (10.3%)	15 (51.7%)	11 (37.9%)	0.018
No	31 (51.6%)	1 (3.2%)	7 (22.5%)	23 (74.1%)	

Receiving a training about dairy cattle feed management and aflatoxin contamination has a statistically significant association ($p = 0.018$) with the knowledge level of dairy cattle owners.

5. DISCUSSION

The present study found that high aflatoxin B1 (AFB1) contamination was present in 88.3% of pooled dairy feed samples collected from intensive smallholder dairy farms in the Akaki Kality, Nifas Silk Lafto, and Lemi Kura sub-cities of Addis Ababa. The findings also showed a higher mean AFB1 concentration in the feed samples (276.05 ± 239.74 $\mu\text{g}/\text{kg}$) than that reported in an earlier study from Addis Ababa milk shed areas, which found a mean AFB1 level of 91 ± 124 $\mu\text{g}/\text{kg}$ in all (100%) feed samples (Gizachew *et al.*, 2016). In addition, a similar study from North West Ethiopia reported AFB1 contamination in 96% of samples, with a comparatively lower mean value (88 ± 33.71 $\mu\text{g}/\text{kg}$) than that observed in the current study (Tang *et al.*, 2023). The concentration of AFB1 in all contaminated feed samples in the present study was higher than the maximum allowable limit of 5 $\mu\text{g}/\text{kg}$ set by the EU, as well as 20 $\mu\text{g}/\text{kg}$ established by the Codex Alimentarius Commission (CAC) and the Institute of Ethiopian Standards. This finding is nearly consistent with the studies conducted in the Addis Ababa milk shed areas and Northern Ethiopia (Gizachew *et al.*, 2016).

Several factors may influence the presence of AFB1 contamination in dairy feeds. In the present study, commercial concentrate feeds and oil seed cake detected in pooled dairy feed samples were major contributors to AFB1 contamination ($p \leq 0.05$). This finding is consistent with Gizachew *et al.* (2016) study. It indicates that oil seed cake included in commercial concentrate feed and/or used as part of a total mixed ration on dairy farms is an important factor affecting AFB1 contamination levels in dairy feeds. In addition, the poor feed storage conditions observed in this study that were characterized by wet storage areas, placing feed containing bags directly on the floor, and inadequate ventilation in the feed store, were significant factors associated with AFB1 contamination ($p = 0.0004$). High level of AFB1 contamination was detected in 91.7% of feeds (652.2 ± 300.19 $\mu\text{g}/\text{kg}$) stored under poor conditions than feeds (87.5%) obtained from farms with good storage conditions (177.54 ± 54.87 $\mu\text{g}/\text{kg}$). The contamination of feeds obtained from farms with good storage conditions is may be attributed to prior AFB1 contamination of dairy feed ingredients used in feed processing plants, especially agricultural products during harvest and/or storage (Molnar *et al.*, 2023; Falade *et al.*, 2022; EFISC, 2015).

Studies from Ethiopia and Kenya have likewise reported substantial AFB1 contamination in feed samples obtained from dairy feed manufacturers/processors and traders, which supports this explanation (Gizachew *et al.*, 2016; Kang & Lang, 2009). This suggests that AFB1 contamination may have occurred previously in agricultural and industrial by-products used as dairy feed ingredients by feed manufacturing companies or agricultural producers (Dhakal and Hashmi, 2024).

On the other hand, only a small proportion of raw cow's milk samples (10%) were contaminated with aflatoxin M1 (AFM1). In all contaminated samples, the AFM1 concentration remained below the maximum permissible limit of 0.05 µg/L established by the EU and as well below the 0.5 µg/L limit set by the Codex Alimentarius Commission and the IES. The findings of the present study were much lower than those of a previous study in Ethiopia, which reported AFM1 contamination in all raw cow's milk samples (100%) with a mean concentration of 0.39±0.85 µg/L using the ELISA method (Gizachew *et al.*, 2016). Other studies conducted in Ethiopia also found comparatively higher mean AFM1 contamination levels in raw cow's milk samples using ELISA (0.32 µg/L, 0.690 µg/L) and HPLC (0.047 µg/L) methods (Kebede *et al.*, 2024; Zebib *et al.*, 2022; Tadesse *et al.*, 2020). Similarly, higher AFM1 levels have been reported in raw cow's milk samples from Kenya, Brazil, and Bangladesh (Tarannum *et al.*, 2020; Kang & Lang, 2009; Sassahara *et al.*, 2005).

The variation in AFM1 contamination results in raw cow's milk may be attributed to differences in the analytical methods (ELISA) used by the above-mentioned authors. However, studies that used HPLC, which has comparatively higher sensitivity, have also found AFM1 levels greater than those observed in the present study (Geleta *et al.*, 2024; Kebede *et al.*, 2024). Nevertheless, the relatively low AFM1 contamination and concentration in the raw cow's milk samples may be explained by the more recent and sensitive detection method (Ultra-HPLC) applied in the present study.

In the present study, all AFM1-contaminated milk samples came from farms whose feeds were contaminated with AFB1, although not all AFB1 positive feeds caused the excretion of AFM1 in the respective milk samples obtained from the same farms. This could possibly be due to low carry-over rate (variation in AFB1 metabolism), low feed intake of contaminated feeds or ELISA overestimation of AFB1 in the feeds. Several studies from different regions have reported that AFM1 in raw cow's milk is linked to AFB1 contamination in dairy cow feeds (Alvarez *et al.*, 2022; Admasu *et al.*, 2021; Kerekes, 2019; Kim *et al.*, 2010). This indicates that appropriate feed management can help prevent or reduce AFB1 contamination in dairy feeds and the resulting AFM1 contamination in cow's milk, thereby helping protect humans from AFM1 exposure.

All dairy cattle owners (100%) were familiar with mold and the conditions that support its growth in feed and human foodstuffs. In contrast, only small proportion (10%) is aware of aflatoxin contamination in dairy feeds and human foodstuffs. This result agrees with the survey findings of a study by Kebede and co-authors (Kebede *et al.*, 2024). Likewise, a study from Pakistan found that 95% of the dairy farmers involved had never heard the term aflatoxin in relation to feed and cow's milk (Yunus *et al.*, 2022). In addition, only 8.3% of the dairy cattle owners in the present study knew that cow's milk can be contaminated by aflatoxins and considered contaminated dairy feed as a possible source. All of the dairy cattle owners in this study believed that eating moldy feeds and foodstuffs could affect the health of both dairy cattle and humans, although they were unable to specify the related public health risks. In addition, they had limited knowledge of the immediate and long-term health effects associated with aflatoxins. Majority of the respondents (80%) have at least primary education. However, the overall knowledge level of most respondents (56.6%) regarding aflatoxins was low. Furthermore, having received training in feed management showed a statistically significant association ($p = 0.018$) and was more important than attaining a higher level of education ($p = 0.64$).

Although respondents were well informed about the conditions that favor mold growth in feed and the associated health risks, the actual feed storage practices in their store were poor in 20% of cases. In contrast, good practical feed storage conditions were observed in 8.33% of dairy farms where owners had strong knowledge of aflatoxin contamination in feeds. This highlights the need to increase dairy farmers' awareness of mold development and aflatoxin formation in feed, as well as contamination of cow's milk with aflatoxin. The authors of a study carried out in Tanzania under the "Tanzania Initiative for Prevention of Aflatoxins Contamination" reported that the awareness program introduced in some regions through extension officers at village meetings was responsible for the greater awareness of aflatoxins in animal feeds and milk (Onesmo *et al.*, 2024).

6. LIMITATIONS OF THE STUDY

The analytical procedures applied in this study for dairy feeds and raw cow's milk was not identical, although comparable methods would have been preferable for correlation analysis. This discrepancy resulted from limitations in laboratory consumables, particularly immunoaffinity columns coated with AFB1 and AFM1. In addition, the present study did not focus on feed processing companies that use agricultural and industrial by-products to produce commercial dairy feeds for smallholder dairy farms in the city. Including these actors would have helped definitively identify the exact source of AFB1 contamination along the value chain. Moreover, although we attempted it, this study did not include analysis of human urine samples to determine the presence of AFM1.

7. CONCLUSION AND RECOMMENDATIONS

A high level of AFB1 contamination was found in pooled dairy cattle feed collected from smallholder dairy farms in Addis Ababa, with all positive samples exceeding the maximum limits established by Codex and the IES. The study showed that AFB1 contamination in dairy feed is linked to poor feed storage conditions on dairy farms. Oil seed cake and commercial concentrate feed were important risk factors. In contrast, the extent of contamination and the AFM1 concentration in raw cow's milk samples were very low. None of the milk samples surpassed even the more stringent EU maximum permissible limit. All AFM1 contamination detected in cow's milk was associated with AFB1 contamination in dairy feed. Although dairy cattle owners have good knowledge of mold growth and the conditions that promote it, their limited awareness of aflatoxin contamination in dairy feeds and cow's milk indicates the need for training on the basic aspects of aflatoxin contamination in dairy feeds, its transfer into cow's milk, and the possible health risks for dairy cattle and human consumers.

Based on the above conclusion, the following recommendations are forwarded:

- Dairy cattle owners should adopt safe feed-handling practices on their farms, such as storing feeds in a dry, well-ventilated, separate store.
- Basic training should be provided to dairy cattle owners by different stakeholders such as universities and NGOs on good feed handling practices.
- Awareness of aflatoxin should be included into existing agricultural extension programs.
- Dairy cattle owners whose feeds show no AFB1 or only low levels of contamination should continue using safe feed handling practices.
- Further research should be conducted on the evaluation of AFB1 in dairy cattle feeds and feed ingredients at different stages or checkpoints along the value chain, as well as the detection of AFM1 in cow's milk using similar detection techniques for both feed and milk.

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ANNEXES

ANNEX I: Feed sample preparation and assay procedure for the detection of Aflatoxin B1 using ELISA technique

- Grind the feed sample until it reaches the particle size of fine instant coffee, with 95% passing through a 20-mesh screen.
- Prepare the extraction solvent (80% acetonitrile or methanol) by combining 40 ml of distilled or deionized water with 160 ml of acetonitrile for each sample to be analyzed.
- Place 2 g of the ground sample into a container and add 200 ml of 80% acetonitrile. Note: the sample-to-extraction-solvent ratio is 1:100 (w/v).
- Shake the sealed container for at least 10 minutes to mix thoroughly.
- Centrifuge the sample at 3,500 rpm for 5 minutes to sediment the particulate matter.
- Collect the supernatant that contains aflatoxin for analysis.
- Dilute an aliquot of the extract 1:10 in reconstituted wash buffer.
- The sample is now ready for testing.
- The final dilution used in calculations is 1:1000.

Assay Procedure:

- Allow all reagents to reach room temperature before use.
- Prepare the wash buffer by dissolving the PBS-T powder packet, rinsing the contents into a 1-L container with a gentle flow of distilled or deionized water.
- Bring the volume to 1 L with distilled or deionized water, and keep refrigerated when not in use.
- Place one mixing well in a microwell holder for each standard and sample to be analyzed.
- Place twice that number of antibody-coated microtiter wells in a separate microwell holder. If using single wells, adjust the volumes accordingly.
- Return any unused wells to the foil pouch with desiccant and reseal it.
- Mix each reagent by gently swirling the reagent bottle before use.
- Dispense 200 μ L of the Assay Diluent into each mixing well.
- Note: For infant or toddler milk formula samples, use the modified assay diluent supplied separately (Product No. ASY5010). (Shake the bottle well before use. Use the modified assay diluent for the standards ONLY). For unknown samples, use the sample diluent already

supplied with this kit.

- Using a new pipette tip for each addition, transfer 100 μL of each standard and prepared sample into the appropriate mixing well containing diluent.
- Mix by priming the pipettor at least three (3) times. Note: The operator should document the position of each standard and sample during the entire test.
- With a fresh pipette tip for each transfer, move 100 μL from each mixing well into the matching antibody-coated microtiter well. Leave at room temperature for 30 minutes.
- Note: The mixing wells contain enough solution to test each standard and/or sample in duplicate, which is recommended. If each standard or sample is tested in singlet or if additional replicates are required, adjust the volumes of assay diluent and sample/standard proportionally.
- Pour the contents of the microwells into a waste basin. Wash the microwells by filling each one with PBS-T wash buffer and then pouring the wash solution into a waste basin.
- Repeat this wash step until a total of three (3) washes has been completed.
- Tap the microwells face down on a layer of absorbent towels to remove any remaining wash buffer.
- Add 100 μL of aflatoxin HRP-conjugate to each antibody-coated well and incubate at room temperature for 30 minutes. Keep covered to protect from direct light.
- Repeat steps 6 and 7.
- Measure the needed volume of substrate solution (1 mL/strip or 120 μL /well) and transfer it to a separate container. Add 100 μL to each microwell. Incubate at room temperature for 10 minutes. Keep covered to protect from direct light.
- Measure the necessary amount of stop solution (1 mL/strip or 120 μL /well) and transfer it to a separate container. Add 100 μL to each well in the same order and at the same rate as the substrate solution was dispensed.
- Read the optical density (OD) of each microwell using a microtiter plate reader with a 450 nm filter, and record the OD for each microwell.
- Using the zero standard as 100% binding (B_0), calculate % binding (%B) for each standard and sample as a percentage of the zero binding (%B/ B_0).

ANNEX II: Determination of AFM1 in milk by HPLC with immunoaffinity column clean-up (AOAC Official Method 2000.08)

PROCEDURES

Sample preparation: Warm the milk to 37 °c in a water bath before analysis, then mix it gently with a magnetic stirrer to disperse the fat layer. Centrifuge the liquid milk at 4000 rpm for 10 min to separate the fat, and remove the thin upper fat layer. Filter the sample through one or more paper filters, collecting at least 50 mL.

Conditioning of immunoaffinity columns: Allow the immunoaffinity columns to come to room temperature. Follow the manufacturer's instructions for column conditioning. Ensure that a small volume (0.5 mL) of PBS remains above the column until the sample solution is applied.

Immunoaffinity clean-up: Load 50 mL of the filtered milk onto the column and allow it to pass by gravity at a flow rate of about 3 mL/min (approximately 1-2 drops per second), then discard the eluate. Rinse the column with 2×10 mL of water at a maximum flow rate of 5 mL/min, discard the eluates, and dry the column. After thorough washing, blow the column to dryness. Elute the aflatoxin slowly with 1.0 mL of methanol, allowing it to pass by gravity, and collect the eluate in a conical tube. Wait 1 min, then apply a second 1.0 mL portion of methanol. Evaporate the eluate to dryness with N stream. Reconstitute the dried residue in 500 μ L of mobile phase (acetonitrile: methanol: water, 25:10:65 v/v).

ANNEX III: Questionnaire

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Parasitology and Poultry health

Dear respondents, the purpose of this questionnaire is to evaluate the knowledge, attitude, and practices of smallholder dairy farm owners regarding mold and aflatoxin contamination. Your responses will be kept confidential and used only for research purposes.

Section I: Demographic Information

- i. Age: _____
- ii. Gender: Male: Female:
- iii. Education Level: No formal education: Primary: Secondary: Tertiary:
- iv. Occupation: _____ v. Duration in dairy farming activity: _____

Section II: Knowledge towards mold growth and aflatoxin contamination

1. Have you heard of mold growth in animal feeds? Yes: No:
2. Have you heard of aflatoxin production in feeds? Yes: No:
3. Have you heard of mold growth in human foods? Yes: No:
4. Have you heard of aflatoxin production in foods? Yes: No:
5. If yes, how did you learn about aflatoxin? (Select all that apply)
Media (TV, radio, internet): Community meetings: Trainings:
Educational institutions: Extension officers: Other (specify): _____
6. What do you think are the causes of mold growth/aflatoxin in animal feeds?
Using contaminated agricultural by-products/ingredients: Poor storage condition:
Other (please specify): _____ I don't know:
7. What do you think are the favorable conditions for mold growth on feed and toxin formation?
Moisture: Warm T^o: Nutrient availability: Extended Storage:
Lack of airflow (poor ventilation): Don't know:
8. Have you heard about aflatoxin in cow's milk? Yes: No:
9. What do you think are the causes/sources of aflatoxin contamination in cow's milk?
AFs contaminated feeds: Other (please specify): _____. Don't know:
10. What do you think are the causes/sources of aflatoxin contamination in humans? (Select all that apply)
Milk: Plant origin foods: Other (please specify): _____. Don't know: ____

Section III: Attitudes related to mold growth and aflatoxin contamination

- 11. Do you think that mold causes disease in animals? Yes: No:
- 12. Do you think that aflatoxin causes disease in animals? Yes: No:
- 13. Do you think that mold causes disease in humans? Yes: No:
- 14. Do you think that aflatoxin causes disease in humans? Yes: No:

Section III: Practices related to feed management and aflatoxin contamination

- 15. Do you check for the growth of mold in dairy feeds? Yes: No:
- 16. How does the dairy feed storage condition in your farm looks like? It is...
Dry area: Cool: Enough ventilation: Raised feed laying-wood board:
- 17. For how long do you store feeds in your store? _____
- 18. Do you fed your dairy animals with a moldy feed? Yes: No:
- 19. Do you consume a **food** on which mold is grown? Yes: No:
- 20. Have you attended any training or workshops on dairy feed management, food safety and aflatoxin contamination? Yes: No:
- 21. If yes, please describe the training/workshop: _____

Field observations during feed/milk sample collection from dairy farms:

Farm ID: _____

- 1. The type of feed commonly used to feed dairy cows?
Concentrate: ___ Wheat bran/Furushka: ___ Oil seed cake: ___ Brewery by-product: ___
Bean/Pea hull: ___ Hey: ___ Silage: ___ Grass: ___
- 2. Feed storage/management conditions in the farm (wet/dry, enough ventilation/not, Raised feed laying-wood board): _____

ANNEX IV: Informed Consent Form for Participation in Research Study

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Parasitology and Poultry health

Title of study: Detection and Evaluation of Aflatoxin Contamination in Feed and Milk with Assessment of Knowledge, Attitude and Practices of Dairy Cattle Owners towards Aflatoxins contamination in Addis Ababa, Ethiopia

Purpose of the Study:

Dear dairy farm owners and workers, you are being invited to participate in a research study aimed at detecting and evaluating the levels of aflatoxin contamination in dairy feeds and cow's milk in selected sub-cities of Addis Ababa area. Your participation involves:

Feed and Milk Sample Collection: You will be asked to provide a 500g feed and 100ml cow milk sample, which will be collected in a sterile container. This sample will help analyze the presence of aflatoxins.

Questionnaire: You will be asked a reply to a questionnaire regarding your knowledge and practices related to dairy feed storage and handling and aflatoxin contamination.

Confidentiality: All information collected will be treated confidentially and used only for research purposes. Your identity will not be released in any reports or publications.

Potential Risks:

- There will be no or minimal risks associated with providing the above samples and answering questions. But, if you feel uncomfortable at any time, you may decline to participate or withdraw from the study without any consequences.

Benefits:

- Your participation will contribute to important research that may help improve animal feed and food safety as well as the awareness in your community.

Voluntary Participation:

- Your participation in this study is entirely voluntary.
- By signing this form, you agree to participate in this study

ANNEX V: Field and Laboratory Pictures

SECTION I: Photo captured during sample and questionnaire data collection



SECTION II: Laboratory analysis (extraction) of feed samples



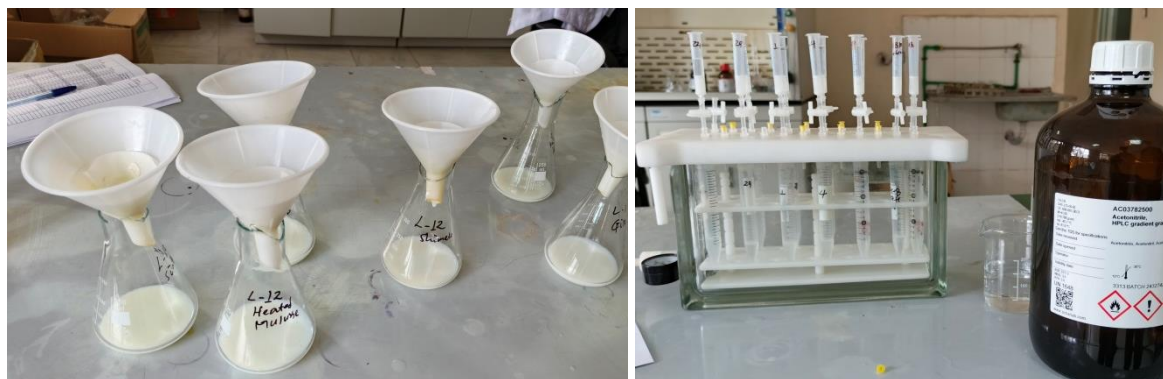
SECTION III: Laboratory analysis (detection of AFB1 by ELISA) of feed samples



SECTION IV: Picture showing microtiter plate (ELISA) reader output

BioTek Instruments												
Assay: AFLA-TOXIN				Date: 08/02/25				Lot: _____				
Wave length: 450, 630				Time: 03:05:32AM				Operator: _____				
Plate ID: CHERE...01												
COMMENTS												
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1											
CALL	2.267	2.446	1.984	2.233	2.009	1.940	2.217	2.006	0.315	0.316	2.162	2.003
CalcOD	STD1	STD1	SMP3	SMP3	SMP11	SMP11	SMP19	SMP19	SMP27	SMP27	SMP35	SMP35
Well	RSLT											
B	S2											
CALL	1.963	2.480	0.935	1.166	2.142	2.042	1.861	1.742	1.996	2.014	1.908	1.932
CalcOD	STD2	STD2	SMP4	SMP4	SMP12	SMP12	SMP20	SMP20	SMP28	SMP28	SMP36	SMP36
Well	RSLT											
C	S3											
CALL	1.672	2.245	0.691	0.913	1.773	1.805	2.019	2.091	1.868	2.003	2.034	1.851
CalcOD	STD3	STD3	SMP5	SMP5	SMP13	SMP13	SMP21	SMP21	SMP29	SMP29	SMP37	SMP37
Well	RSLT											
D	S4											
CALL	1.245	1.705	1.738	2.130	1.776	1.927	2.174	2.152	1.781	2.040	1.994	1.888
CalcOD	STD4	STD4	SMP6	SMP6	SMP14	SMP14	SMP22	SMP22	SMP30	SMP30	SMP38	SMP38
Well	RSLT											
E	S5											
CALL	0.477	0.713	2.107	2.587	1.976	1.928	2.074	2.031	1.919	2.100	1.961	1.894
CalcOD	STD5	STD5	SMP7	SMP7	SMP15	SMP15	SMP23	SMP23	SMP31	SMP31	SMP39	SMP39
Well	RSLT											
F	S6											
CALL	0.242	0.222	2.205	2.590	1.550	1.637	2.184	2.140	2.034	2.154	2.075	1.980
CalcOD	STD6	STD6	SMP8	SMP8	SMP16	SMP16	SMP24	SMP24	SMP32	SMP32	SMP40	SMP40
Well	RSLT											
G	S7											
CALL	2.010	2.494	0.270	0.270	1.498	1.527	2.246	2.157	2.021	2.165	2.123	1.992
CalcOD	SMP1	SMP1	SMP9	SMP9	SMP17	SMP17	SMP25	SMP25	SMP33	SMP33	SMP41	SMP41
Well	RSLT											
H	S8											
CALL	0.285	0.247	1.922	2.227	1.317	1.683	2.200	2.021	2.123	2.085	2.026	2.022
CalcOD	SMP2	SMP2	SMP10	SMP10	SMP18	SMP18	SMP26	SMP26	SMP34	SMP34	SMP42	SMP42
Well	RSLT											

SECTION V: Laboratory analysis (extraction) of milk samples



SECTION VI: Laboratory analysis (detection AFM1 by UHPLC) of milk samples



ANNEX V: Ethical Clearance Certificate

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Research Ethics Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/04/119/17/2026

Name of Applicant: **Charnet Balcha Jima (MSc Student)**

Address: Department of Microbiology, Parasitology and Poultry Health, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Detection and Evaluation of Aflatoxins Contamination in Feed and Milk with the Assessment of Knowledge, Attitude and Practices towards Aflatoxin Contamination in Addis Ababa, Ethiopia*

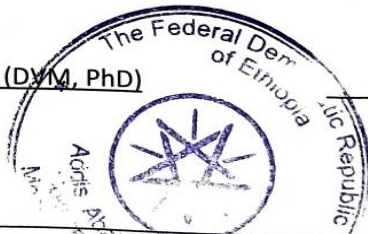
Date of application: **December, 2024**

Nature of the project: **Field investigation**
Target animal species: **Dairy Cattle**
Number of animals involved: **None**
Study area: **Addis Ababa, Ethiopia**

Minutes No. and date of review: **VM/ERC/04/17/025, 25/02/2025**

The Institutional Animal Care and Use Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University has reviewed the above research project and unanimously approved the application of Student Charnet Balcha Jima.

Professor Getachew Terefe (DVM, PhD)
Chairman




Signature

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Please quote Our Ref. No. When replying

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Tel. +251 114338450

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P.o.x. Box}34

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Bishoftu, Ethiopia



Aklilu Lemma Institute of Pathobiology Institutional Research Ethics Review Committee (ALIPB-IRERC)

Ethical Clearance Certificate

Ref. No.: ALIPB IRERC/174/2017/25

Date: February 10, 2025

Title of the project: "Detection and evaluation of aflatoxin contamination in feed, cow milk, and human urine in Addis Ababa and its surrounding, Ethiopia"

PI: Chernet Balcha,
Recommendation of the ALIPB-IRERC

Dear: Chernet,

The ALIPB-IRERC has reviewed your above mentioned Research Proposal and noted its merit. The IRERC would like to remind you as the PI to submit progress reports of the work every 6 months and the final report upon completion of the study. Furthermore, you are expected to notify the ALIPB-IRERC ahead of time any amendments or modifications in the protocol or premature suspension or termination of the study.

STATUS: **Approved**

Needs NRERB clearance:

Yes: ___ No: x

IRERC Chairperson: Berhanu Erko, Prof.

Signature: Berhanu Erko

IRERC Secretary: Esayas Aklilu, PhD.

Signature: Esayas Aklilu

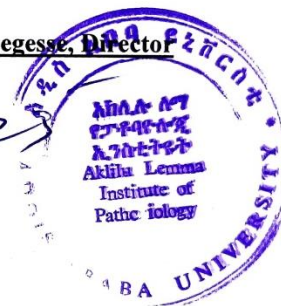
Approval

Name: Professor Mengistu Legesse, Director

Signature: Mengistu Legesse

Date: Feb 10, 2025

Cc// IRERC office



DETECTION AND EVALUATION OF AFLATOXINS
CONTAMINATION IN FEED AND MILK, WITH THE ASSESSMENT
OF KAPs OF DAIRY CATTLE OWNERS TOWARDS AFLATOXIN
CONTAMINATION IN ADDIS ABABA, ETHIOPIA (Chernet B)

ORIGINALITY REPORT

16% SIMILARITY INDEX	9% INTERNET SOURCES	15% PUBLICATIONS	4% STUDENT PAPERS
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PRIMARY SOURCES

1	Qinghua Wu, Alena Jezkova, Zonghui Yuan, Lucie Pavlikova, Vlastimil Dohnal, Kamil Kuca. "Biological degradation of aflatoxins", Drug Metabolism Reviews, 2009 Publication	1%
2	Martina Loi, Antonio F. Logrieco, Tünde Pusztahelyi, Éva Leiter, László Hornok, István Pócsi. "Advanced mycotoxin control and decontamination techniques in view of an increased aflatoxin risk in Europe due to climate change", Frontiers in Microbiology, 2023 Publication	1%
3	Submitted to University of Hong Kong Student Paper	<1%
4	Nan Zheng, Li Min, Dagang Li, Sheng Tan, Yanan Gao, Jiaqi Wang. "Occurrence of Aflatoxin M1 in Cow, Goat, Buffalo, Camel, and Yak Milk in China in 2016", Toxins, 2022 Publication	<1%
5	etd.hu.edu.et Internet Source	<1%
6	Li Min, Dagang Li, Xiong Tong, Hao Sun, Weidong Chen, Gang Wang, Nan Zheng, Jiaqi Wang. "The challenges of global occurrence of aflatoxin M1 contamination and the reduction	<1%