Aflatoxin M1 in Raw Milk Collected from Specialized Dairy Farms and Local Markets in Selected Urban Centers of Eastern Ethiopia

Angassa Tadese Tesfaye * , Mohammed Yusuf Kurtu , Yesihak Yusuf Mume , Abdi Hassan Mohammed

Posted Date: 6 March 2024

doi: 10.20944/preprints202403.0371.v1

Keywords: Aflatoxin M1; Raw milk; Dairy cow; Milk vendors; HPLC; Eastern Ethiopia

Article

Aflatoxin M_1 in Raw Milk Collected from Specialized Dairy Farms and Local Markets in Selected Urban Centers of Eastern Ethiopia

Angassa Tesfaye [1,2,*], Mohammed Kurtu [2], Yesihak Yusuf [2] and Abdi Mohammed [3]

[1] Department of Animal and Range Science, College of Agricultural Sciences, Bule Hora University, Bule Hora, Ethiopia
[2] School of Animal and Range Science, College of Agriculture and Environmental Science, Haramaya University, Maya, Ethiopia; mohamedkrt7@gmail.com; yesihakyus@gmail.com
[3] School of Plant Science, College of Agriculture and Environmental Science, Haramaya University, Maya, Ethiopia; abdi.mohammed22@yahoo.com
* Correspondence: angassatesfaye@gmail.com

Abstract: Aflatoxin M_1 (AFM_1), a carcinogenic toxin, mainly contaminates cow milk and poses significant challenges to the dairy industry, thereby, public health in most tropical countries, including Ethiopia. This study aimed to investigate the prevalence and level of AFM_1 in raw cow milk collected from Specialized Dairy Farms and Milk Vendors in three Urban Centers: Chiro town, Dire Dawa, and Harar cities in Eastern Ethiopia. 180 milk samples were collected from two major Milk Sources: Specialized Dairy Farms (N=90) and Milk Vendors (N=90) using a simple random sampling technique. AFM_1 was analyzed using high-performance liquid chromatography. The study revealed that, 63.9% (115/810) prevalence and 0.179±0.48µg/L mean of AFM_1 level in milk, which 39.13% and 26.08% exceed the tolerable limits of EU and FDA respectively. A 40.0% prevalence and 0.344±0.72µg/L average of AFM_1 in the milk from Dire Dawa city was significantly higher than the samples from other Urban Centers. However, 30.43% and 29.57% prevalence as well as 0.055±±0.13µg/L and 0.140±0.33µg/L average of AFM_1 in milk from Chiro town and Harar city, respectively were not significant. Furthermore, milk samples from Dairy Farms revealed a higher prevalence (57.39%) and level (0.252±0.64µg/L) of AFM_1 than Milk Vendors, with prevalence of 42.61% and average, 0.107±0.21µg/L. Therefore, this study concludes that substantial milk samples were contaminated by AFM_1 and urges the need to enhance farmers' awareness on mitigation.

Keywords: aflatoxin M_1; raw milk; dairy cow; milk vendors; HPLC; Eastern Ethiopia

1. Introduction

Mycotoxins are low molecular weight secondary metabolites, produced by toxigenic fungi during their morphological and chemical differentiation, and are highly prevalent in many foods and feedstuffs [1]. Currently, of over three hundred types of mycotoxins uncovered, aflatoxin is the most potent carcinogenic agent, threatening humans, and animal health, and primarily produced by *Aspergillus flavus* and *Aspergillus parasiticus* [2,3]. Specifically, four types of aflatoxins: B_1, B_2, G_1, and G_2, are known to be produced by Aspergillus species [4,5]. However, aflatoxin M_1 (AFM_1) is the hydroxylation byproduct of aflatoxin B_1 in the liver of lactating dairy cows that ingested the contaminated feedstuffs [3,6].

Milk is the main food in many countries, due to its valuable source of nutrients, where milk from dairy cows shares a significant contribution. Globally, 81 percent or 746 million tons of milk has been produced by dairy cows in 2021 [7]. Similarly in Africa, 77% of the total milk were come from dairy cows [8]. Specifically, of a total of 7.12 billion liters of milk produced in Ethiopia in 2020/21, 65.84% or 4.69 billion liters of milk were accounted as dairy cow's milk [9]. However, the presence of AFM_1 in the milk of dairy cows raises serious concerns of food safety and is usually, linked to cancer diseases, hepatotoxins, and others in humans and animals [3,10]. Subsequently, the International

 doi:10.20944/preprints202403.0371.v1

Agency for Research on Cancer [11], has classified AFM_1 as Group 2B carcinogenic agent. Moreover, the association of a lasting adverse health condition (like child stunting) with children consuming AFM_1-containing milk, elevates its public health importance, where milk is widely regarded as a complementary diet for them [10,12]. The primary animal exposure route of aflatoxins is through ingestion of frequently contaminated crops, such as cereal grains, oilseeds, nuts, or others that are integral parts of feed ingredients [13,14]. While, human exposure to AFM_1, is mainly, through the consumption of contaminated milk and milk products [14].

The proliferation of Aspergillus fungi and associated aflatoxins are determined by the host, fungus, and environmental stresses interaction [1]. Thus, Medina et al., [15] indicated that hot temperature and high moisture/humidity increase aflatoxin excretion, by modulating aflatoxin synthesizing genes. The hot and humid climates, with mean annual rainfalls >700 mm and relative humidity >60% in tropical and subtropical climates provide a conducive environment for Aspergillus proliferation, and concomitant aflatoxin M_1 in milk [3]. Additionally, pre- and postharvest management practices such as cropping system, time of harvest, stage of dryness, storage conditions, and transportation have significantly contributed to fungal development in feeds and ultimately, lead to aflatoxins contamination in feed and milk [16–18].

Thus numerous studies in sub-Saharan African countries with warm-humid tropics and subtropics climates were highly prevalent with AFM_1 in the raw milk of dairy cows. Accordingly, Kagera et al., [19] reported, 98.8% (N=84) prevalence and 83.66±64.68 ng/L mean of AFM_1 in raw milk collected from the smallholder dairy farmers in the Kasarani sub-county of Kenya. In the same country, a higher prevalence of 100%, (N=150) was reported with 58% of the sample exceeding the EU tolerable limit [20]. However, Anyango et al., [21] reported, a comparably lower occurrence of AFM_1 (37.5%, N=72) in raw milk collected from the urban and peri-urban areas of Kisumu county in Kenya, which 26.4% exceed the tolerable limit of EU standard.

Similarly, a 100% (N=112) prevalence and 0.55±0.18 µg/L level of AFM_1 was reported in raw milk of dairy cows collected from three agro-ecological zones in Malawi, which 98% and 22% of the sample exceeded the tolerable limit of EU and FDA, respectively [22]. Of 25 milk samples collected from Dairy Farms in Khartoum, 92% contain AFM_1 [23]. Likewise, Nishimwe et al., [24] reported, a 91.8% (N=170) prevalence, and 0.89±1.64µg/L mean of AFM_1 with a 38.8% sample exceeding the EU tolerable limit in Rwanda. Also, a high prevalence of AFM_1 (83.8%, N=37) was reported in milk collected from smallholder dairy farmers in Tanzania, where 100% and 16.1% exceeded the EU and FDA tolerable limits, respectively [25]. While in a recent finding by Kitigwa et al., [26], a lower prevalence of 30.7% with 27.9% exceeding the EU limit, was reported in milk samples collected from the smallholder dairy farmers in Tanzania.

Like in many East African countries, research revealed that AFM_1 posed a critical challenge for the dairy industry, with the significant prevalence in raw milk of dairy cows in Ethiopia. Accordingly, Dawit et al., [27] reported, a 100% prevalence and 4.91 µg/L average of AFM_1 in 110 raw milk collected from the greater Addis Ababa milk sheds, which 97.8% of the sample exceeded the EU tolerable limit of 0.05 µg/L, and 26.3% surpassed the FDA limit of 0.5 µg/L. A year later, a study conducted by Abenet [28] revealed that 93% (N=42) of milk samples from Dairy Farms in Addis Ababa and nearby districts had been contaminated by AFM_1, with 86% of the samples exceeding the EU tolerable limit. In another study conducted in the Guraghe zone of the SNNP region, 80% (N=10) of occurrence and 0.31 µg/L level of AFM_1 in milk samples was reported, where 68% of the samples exceeded the EU tolerable limit of 0.05 µg/L [29].

Furthermore, among 64 milk samples collected from different urban and peri-urban areas of Oromia, Amhara, and SNNP regions all samples were contaminated by AFM_1, with 50% and 14% exceeding the EU and FDA limit respectively [30]. Similarly, a 100% (N=108) prevalence and level of 0.69±0.505 µg/L of AFM_1, in which 96% and 82% of the samples exceeded the EU and FDA limits respectively, was reported in raw milk collected from Bishoftu town in Ethiopia [31].

In Ethiopia, the urban population has been projected to be 23.20% by 2023, signifying rapid urbanization [32], with a 4.8% rate of urbanization increase annually [33]. Derived by rapid urbanization, then, the demand for milk and milk products has been increasing in the major Urban

 doi:10.20944/preprints202403.0371.v1

Centers [33]. Thus, to address these demand-supply gaps for milk and milk products in urban areas, the roles played by urban and peri-urban dairy and specialized dairy production systems (SD) are remarkable [33]. Thus, Shapiro et al., [34] reported, a 125% increase in milk production by the SD from 2014/15 to 2019/20, where these specialized dairy and most urban and peri-urban dairy, primarily rely on concentrate supplementation [35–37].

Likewise, in this study, the three selected Urban Centers of Eastern Ethiopia, the rapidly increasing urban dwellers, contributed to the demands for milk and milk products [38][39][40]. Consequently, substantial indoor dairy operations, where wheat bran, maize feeds, total mixed rations, and brewer's yeast byproducts were commonly supplemented to the dairy cows to increase milk yield [41,42]. However, the high susceptibility of these feeds to the Aspergillus species and associated aflatoxins [27], and consequent led to contamination of milk, poses a critical challenge to the dairy industry as well as public health [43]. Therefore, this study aimed to investigate the prevalence and level of AFM_1 in raw milk collected from indoor Dairy Farms and local Milk Vendors across the three selected Urban Centers in Eastern Ethiopia.

2. Materials and Methods

2.1. Study Site Selection

This study was conducted in three major Urban Centers of Eastern Ethiopia such as Chiro town, Dire Dawa, and Harar cities (Figure 1). These Urban Centers were purposively selected for their potentiality of the specialized dairy farming and main milk sheds/marketing centers of the region [39,41,42]. The selected urban centers are situated in varied agro-ecological climates. Thus, Chiro town has semi-arid agro-ecology (1,757 m.a.s.l.) [44], while Dire Dawa city is situated in lowland agro-climates (1170 m.a.s.l.) [45]. However, Harar city is located within a range of 1900-2200 m.a.s.l. where most parts have highland agro-climates [46].

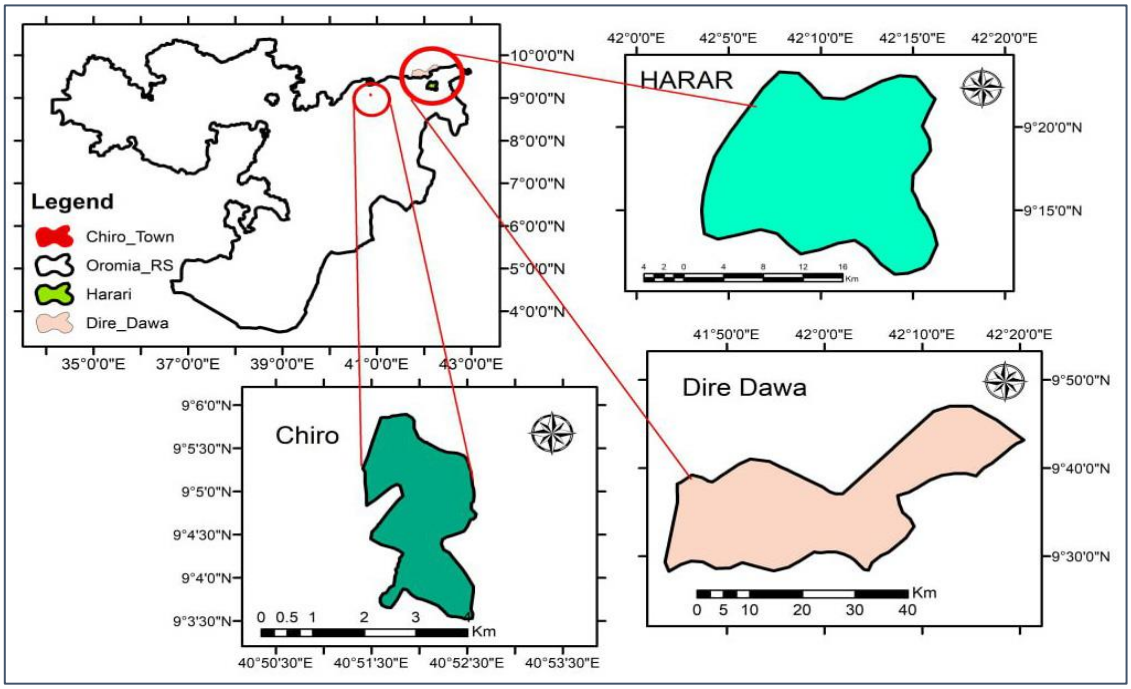

Figure 1. Map of the study areas.

2.2. Milk Sample Collection

In this research, raw milk samples of dairy cows were collected from specialized dairy farms and milk vendors in Chiro town, Dire Dawa, and Harar cities in Eastern Ethiopia from September 2021 to January 2022. As a result, a total of 180 milk samples; in which 60-samples per each Urban

 doi:10.20944/preprints202403.0371.v1

Center (Chiro town=60, Dire Dawa city=60 and Harar city=60) were collected. Subsequently, these milk samples were further categorized into two major Milk Sources, such as Specialized Dairy Farms (30) and Milk Vendors (30). Accordingly, 30 raw milk samples were collected from each milk source per urban center. Therefore, a total of 180 (30x2x3=180) raw milk samples of dairy cows were collected and analyzed. The sample size was determined, according to Daniel and Cross, [47], with 0.866 expected prevalence of AFM_1 in milk as reported on [48] and a 5% precision level.

Before milk sample collection, detailed consultations were held with the agricultural administrators, livestock experts, and extension workers of the targeted urban centers to identify the potential kebeles (the smallest administrative unit) for Specialized Dairy farming and major milk marketing centers. Then, the livestock development office provided, a list of specialized dairy farms, from which the sampling dairy farms were randomly selected and used for milk samples collection. Subsequently, in collaboration with livestock extension workers, the local milk vendors were randomly selected by visiting the identified marketing centers, by maintaining equal chances of selection for all attendants at the time of sample collection. Thus, raw milk samples of cow were purchased from the identified milk vendors until the targeted number of samples was obtained.

Thus 0.5 L of milk sample was collected from the bulk of milk containers from each of the identified Dairy farms and Milk vendors into the sterilized and labeled Falcon tube. The milk samples were transported to Haramaya University's Dairy Sciences Laboratory and kept at a -20 ℃ deep freezer until further analysis. AFM_1 analysis was conducted, at the Animal Products, Veterinary Drug and Feeds Quality Assessment Center (AVDFAC) lab facility in Addis Ababa.

2.3. Analysis of Aflatoxin M_1 Using HPLC

2.3.1. Milk Sample Extraction

The liquid milk samples were extracted following the procedures outlined in the AOAC official method [49] with slight modifications. Liquid milk samples retrieved from the deep freezer were placed in a 35-40 ℃ water bath for about 30 minutes. Samples were then manually shaken for 5 min to ensure homogeneity. Followed by centrifugation at 3500 rpm for 15 min, the fat layer was separated and discarded using a spatula. The fatless samples were filtered using Whatman No. 5 filter paper and 50 mL of filtrate was transferred to the cleanup step.

2.3.2. Sample Cleanup and HPLC Condition

The liquid milk sample cleanup for AFM_1 testing was performed according to the instructions enclosed with the test kit of immunoaffinity column (IAC) and the method described by Iqbal et al., [50], with little modifications. Thus, 50 mL of defatted milk samples were passed through the AflaClean IAC (AflaClean LCTech GmbH, Germany) attached to a manifold (Supelco Visiprep™, Germany) and vacuum pump (Primatec, Itu, Brazil) at a flow rate of 1-3 mL/min. The column was washed twice with 10 mL of distilled water at the same flow rate. Then, the bound of AFM_1 was eluted with 3.0 mL of HPLC grade acetonitrile, and the eluate was collected in glass vials. The eluate of AFM_1 residue was evaporated to near dryness using a gentle nitrogen stream at 40 ℃ (MultiVap54, LabTech, Germany). Then, the dried residues were placed in a dark place for 15 minutes at room temperature and the caps of the vials were tightened. Then, a 200 µL of water: acetonitrile: methanol solution (65:20:15, *v*/*v*/*v*) was added to the vials. A 20 µL portion of the solution was injected into the HPLC for AFM_1 analysis.

The Agilent 1200 system (Agilent Technologies, Santa Clara, CA, USA) High-Performance Liquid Chromatography (HPLC) coupled with Fluorescence Detector (FLD), Chemstation Software (Agilent Technologies), binary pump, vacuum degasser, autosampler, and Agilent column (Eclipse XDB-C18, 1.8 µm, 4.6 × 50 mm) was used to analyze AFM_1. The mobile phase is composed of an isocratic mixture of water/acetonitrile (75:25, *v*/*v*) and a flow rate of 1.4 mL/min. The 365 nm excitation and 425 nm emission wavelengths were used.

2.4. Data Validation

 doi:10.20944/preprints202403.0371.v1

Data validation of the method includes linearity, limits of detection (LOD) and quantitation (LOQ), accuracy, and precision as described in the regulation of the EU [51]. The calibration curves to quantify AFM_1 in raw milk were prepared using the AFM_1 standard solutions (Sigma®) prepared in acetonitrile at five levels ranging from 0.01 – 1.0 µg/L. The AFM_1 levels in the samples were linearly correlated with the integrated peak areas of the standard solutions. Thus, the linearity was verified from the calibration curve, and the coefficient of determination (R^2) from regression analysis. The limits of detection (LOD) and limit of quantification were determined from the analysis of the triplicate of the blanks by considering, 3:1 and 10:1 signal-to-noise ratio respectively. The accuracy and precision have been determined by spiking samples at a known concentration levels of AFM_1 standard solution into the test portion of the sample (in triplicate). Then, the accuracy of the method was assessed by the percent of recovery (R %), while precision was expressed as the percent of relative standard deviation (RSD %). Therefore, R% and RSD% were computed using equations 1 and 2.

$$R\% = \frac{\text{Conc. in spiked sample} - \text{Conc. in unspiked sample}}{\text{Conc. in unspiked sample}} \times 100\% \quad (1)$$

$$RSD\% = \frac{\text{SD of replicated concentration}}{\text{Mean of R\%}} \times 100\% \quad (2)$$

2.5. Data Analysis

Data were entered into Microsoft Excel 2016 (MS Excel®) and exported to SPSS software version 27 (IBM SPSS, Chicago, IL, USA) for analyses. Aflatoxin M_1 levels in milk were categorized into legal, beyond legal, or others, based on ESA/EU and FDA acceptance levels of AFM_1. Qualitative data were summarized using graphs and frequency tables. While, mean ±standard deviation, maximum, and minimum values were determined for quantitative data. The significant difference in AFM_1 levels in raw milk between the study site and milk source was determined using the ANOVA test ($\alpha = 0.05$). Also, the regression analysis was used to determine the value of R^2 or the equation of straight line constructed by MS Excel. A significance level of $p < 0.05$ was used.

3. Results

3.1. Method Validation

The linearly relationship between the response of instrument and the concentration of AFM1 was tested plotting a six level standard curve spiked at 0.01, 0.02, 0.04, 0.5, and 1.0 µg/L. Thus Figure 2, demonstrated a linearity with calibration curve equation of y = 0.7779x + 0.0011; where y is the peak areas and x is the concentration of AFM1 (µg/L). Also, the coefficient of determination (R^2) value of 0.9992 revealed an acceptable fit of the data to the regression line.

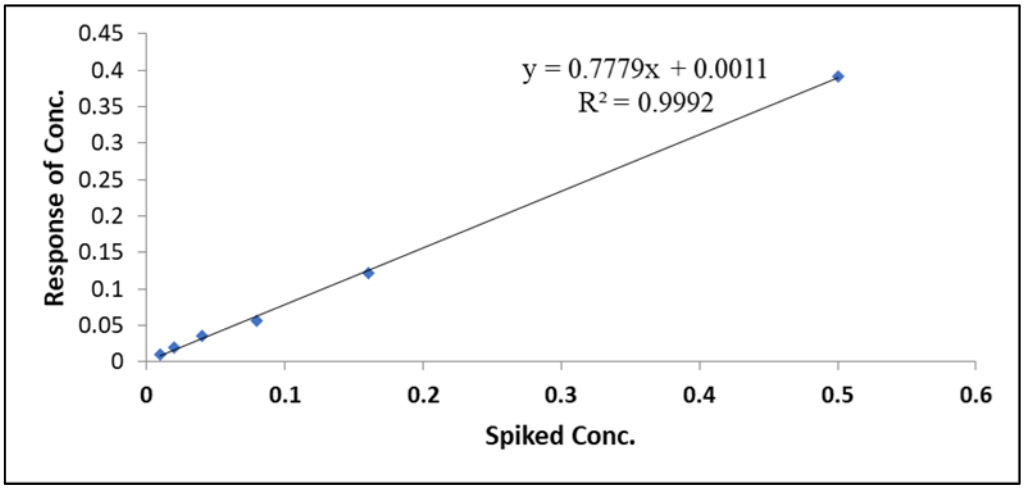

Figure 2. Calibration curve of AFM1 standard.

Preprints.org (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 March 2024 doi:10.20944/preprints202403.0371.v1

Additionally, the sensitivity of the method was evaluated by LOD and LOQ and presented in Table 1. Thus 0.008 µg/L and 0.026 µg/L values of LOD and LOQ were analyzed in the milk samples of this study. The limit of detection and quantification in this study was shown greater sensitivity than the LOD (0.045 µg/L) and LOQ (1.35 µg/L) presented by Adise et al., [52] in raw milk. The result of the accuracy was evaluated percent recovery (%R), while precision was tested by percent of relative standard deviation (%RSD) (Table 1). Thus, the %RSD of the instrument method was 1.71%, while the %R ranges from 127.62–91.64%. The matrix spiked recovery of this study falls between 70 – 120% ranges of guidelines described by the Association Officials of Analytical Chemistry. The percent of relative standard deviation value of the samples was < 5%, indicating that the proposed method was precise.

Table 1. Method performance characteristics for aflatoxins in samples.

Aflatoxins	Spiking level (ppb)	R%	LOD (µg/kg)	LOQ (µg/kg)	RSD%
AFM_1	0.01–0.5	127.62–91.64	0.008	0.026	1.71

LOD = limit of detection; LOQ = limit of quantification; RSD = relative standard deviation.

3.2. Occurrence of Aflatoxin M_1 in Raw Milk across Study Sites and Milk Sources

The prevalence of AFM_1 in raw milk collected from specialized Dairy Farms and local Milk Vendors in three major Urban Centers of Eastern Ethiopia, such as Chiro town, Dire Dawa, and Harar cities is presented in Figure 3. As a result, an overall prevalence of AFM_1 in 115/180 (63.9%) milk samples was found in this study, whereas, 36.1% (45/180) of the samples were negative or below the concentration of LOD (0.008 µg/kg). Also, in all over 180 raw milk analyzed, a higher prevalence of AFM_1 in milk from Dire Dawa city (25.6%) than in milk collected from other Urban Centers was found. Whereas, the occurrence of AFM_1 in milk samples from Chiro town (19.4%) and Harar city (18.9%) were not significantly different. Similarly, all over the milk samples analyzed, the higher occurrence of AFM_1 in milk from the specialized Dairy Farm (36.7%) was found, compared to the occurrence in the samples from local Milk Vendors (27.2%).

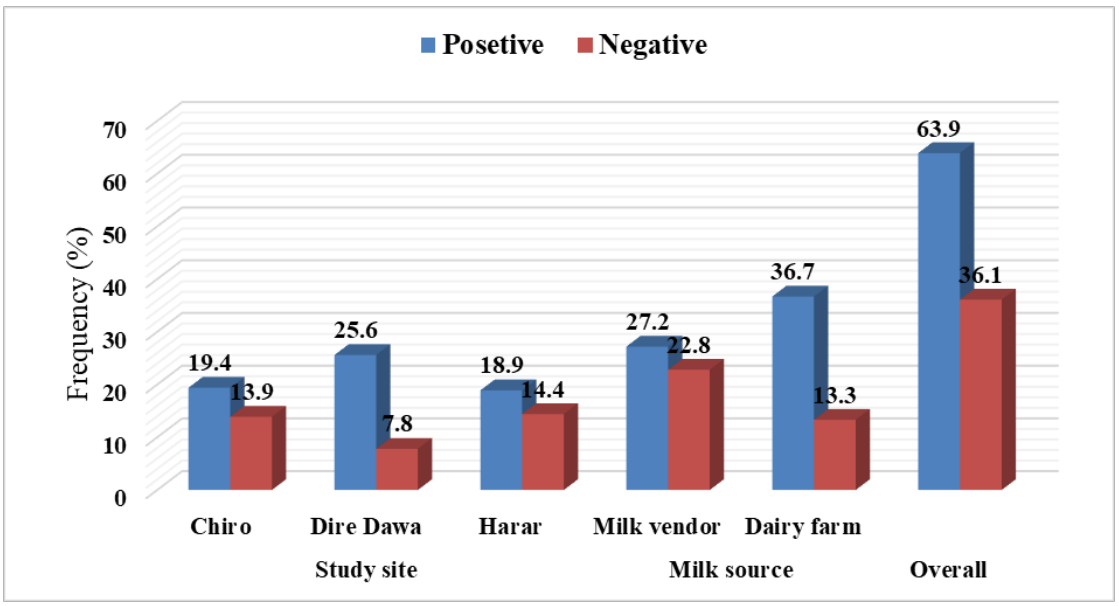

Figure 3. Occurrence of AFM_1 in milk from Dairy Farms and Milk Vendors in Study Sites.

Furthermore, among the contaminated milk samples (N=115), the prevalence of aflatoxin M_1 in raw milk from the specialized Dairy farms and Milk Vendors in the study locations was presented in Table 2. Thus, among the contaminated milk, a substantial number of samples were within the range

 doi:10.20944/preprints202403.0371.v1

of LOD to 0.05 µg/g (60.87%) level of AFM_1, which are in compliant with the 0.05 µg/L maximum permissible limit of the Ethiopian Standard Agency (ESA) [53] or European Union (EU) [54], while a few samples with concentration range of 0.051 to 0.5 µg/kg (13.04%) was found. However, the samples beyond a level of 0.5 µg/kg (26.08%) are critically important.

Moreover, a significant difference ($P < 0.05$) in AFM_1 occurrence, among the contaminated milk samples (N=115) was observed, across the study locations. Thus, a higher prevalence of AFM_1 in the milk samples from Dire Dawa city (40.0%) was found compared to the prevalence in the milk from Chiro (30.43%) and Harar (29.57%) Urban centers. While either AFM_1 levels ranging from LOD to 0.05 µg/L and 0.051-0.5 µg/L were found significant between Study Sites. However, a significantly higher number of samples beyond a 0.5 µg/L, in milk from Dire Dawa city (14.78%), indicated more milk with a higher level of AFM_1 in this Urban Center, than in milk from the other ones.

Similarly, among the positive samples (N=115), the prevalence of AFM_1 in milk from specialized Dairy Farms and local Milk Vendors was found significant ($P < 0.05$). Accordingly, the prevalence of AFM_1 in milk samples collected from specialized Dairy Farms (36.7%) was significantly higher, compared to the prevalence in Milk Vendors (27.2%). Moreover, the concentration range of 0.051 to 0.5 µg/L and beyond 0.5 µg/L was not significant between the Milk Sources, but the concentration ranging from LOD to 0.05 µg/L was significantly higher in the milk collected from Dairy Farms (35.65%) than in Milk Vendors (25.22%).

Table 2. The prevalence of AFM_1 in raw milk across Study Sites and Milk Sources (N=115).

Categories	N+ (%)	LOD-0.05 (%)	0.051-0.5 (%)	>0.5 (%)
Study Sites				
Chiro town	35 (30.43) [a]	26 (22.61)	5 (4.35)	4 (3.48) [a]
Dire Dawa city	46 (40.00) [b]	20 (17.39)	9 (7.83)	17 (14.78) [b]
Harar city	34 (29.57) [a]	24 (20.87)	1 (0.87)	9 (7.83) [a]
P-value	*	**ns**	**ns**	*
Milk Source				
Milk vendor	49 (42.61)	29 (25.22)	7 (6.09)	13 (11.30)
Dairy farm	66 (57.39)	41 (35.65)	8 (6.96)	17 (14.78)
P-value	*	*	**ns**	**ns**
Total	115 (100)	70 (60.87)	15 (13.04)	30 (26.08

Column under the same category that bears different superscripts are significantly different ($\alpha \leq 0.05$); * = P >0.05; ns = not significant; N+ = positive samples; LOD = limit of detection.

3.3. Level of Aflatoxin M_1 in Milk across Study Sites and Milk Sources

The mean level and concentration range of AFM_1 recovered from the raw milk samples collected from Dairy Farms and Milk Vendors across Study Sites were presented in Table 3. Consequently, an overall mean concentration of AFM_1 of 0.179±0.48 µg/L with a range of LOD – 3.85 µg/L was found in this study, in which 39.13% and 26.08% of the contaminated samples exceeded the tolerable limit of Ethiopian Standard Agency (ESA) or European Union (EU) and Food and Drug Authority (FDA) [55] respectively.

Furthermore, the analysis of the mean difference revealed a highly significantly different ($P < 0.01$) concentration level of AFM_1 in milk samples between the Study Sites. Thus, a highly significant mean level of AFM_1 of 0.344±0.72 µg/L within a range of LOD – 3.85 µg/L was recovered in the milk samples collected from Dire Dawa city compared to the mean level found in the samples from Chiro town and Harar city. However, a mean level of 0.055±0.13 µg/L (range: LOD – 0.545 µg/L) in milk

 doi:10.20944/preprints202403.0371.v1

samples collected from the Chiro town was not significant compared to a mean level of 0.140±0.33 µg/L (range: LOD – 1.55 µg/L) in samples collected from the Harar city.

Similarly, 22.61% and 14.78% of milk samples from Dire Dawa city exceeded the maximum limit of ESA/EU (0.05 µg/L) and FDA (0.5 µg/l) levels of AFM_1 respectively, were found significantly higher, compared to the samples exceeding the maximum limit in milk from the other Study Sites. However, the samples greater than the ESA/EU (7.83%) and FDA (3.48%) limit of AFM_1 level in milk from Chiro town was not significant compared to the samples exceeding ESA/EU (8.7%) and FDA (7.83%) limit in milk from the Harar city.

Furthermore, the mean level of AFM_1 concentration recovered from the raw milk was found significant (P >0.05) between the Milk Sources. As a result, a mean concentration of 0.252±0.64 µg/L (range: LOD – 1.037 µg/L) recovered from the milk samples of the specialized Dairy Farms was significantly higher, compared to a mean level of 0.107±0.21 µg/L (range: LOD – 2.701 µg/L) that was recovered from the local Milk Vendors. Also, the samples beyond the tolerable limit of ESA/EU (21.74%) and FDA (14.78%) limit of AFM_1 concentration in milk from specialized Dairy Farms were not significant compared to the samples beyond ESA/EU (17.39%) and FDA (11.30%) limit in milk collected from Milk Vendors. In this study, the interaction of AFM_1 concentration level in milk samples between the Study Sites and Milk Sources was found non-significant (P >0.05).

Table 3. The mean level of AFM_1 in raw milk across Study Sites and Milk Sources.

Category	Mean ±SD (µg/L)	Range (µg/L)	%> ESA/EU (N=115)	%> FDA (N=115)
Study Sites				
Chiro town	0.055±0.13 [a]	LOD – 0.545	9 (7.83) [a]	4 (3.48) [a]
Dire Dawa city	0.344±0.72 [b]	LOD – 3.850	26 (22.61) [b]	17 (14.78) [b]
Harar city	0.140±0.33 [a]	LOD – 1.550	10 (8.7) [a]	9 (7.83) [a]
P-value	**		**	*
Milk Source				
Milk vendor	0.107±0.21	LOD – 2.701	20 (17.39)	13 (11.30)
Dairy farm	0.252±0.64	LOD – 1.037	25 (21.74)	17 (14.78)
P-value	*		**ns**	**ns**
Overall	0.179±0.48	LOD – 3.85	45 (39.13)	30 (26.08)
Study site*Milk source	**ns**	**-**	**-**	**-**

Column mean of AFM_1 levels that bear different superscript are significantly different ($\alpha \leq 0.05$); ns = P >0.05; ** = P <0.01; * = P <0.05; SD = standard deviation; ESA/EU = 0.05 µg/L; FDA = 0.5 µg/L; LOD = limit of detection (0.008 µg/L).

Moreover, the mean level of AFM_1 among the herd size and milk production scale per Dairy Farm were presented in Table 4. Thus, the level of AFM_1 in raw milk among different herd sizes and milk production scales of Dairy Farms was found highly significant (P >0.01). As a result, a highly significant mean level of AFM_1, 0.720±0.975 µg/L (range: 0.017 – 3.850 µg/L) in milk samples corresponding to the Dairy Farm with large herd size (≥16 dairy cows). However, the level of AFM_1 recovered in the milk samples corresponding to medium herd size (6-15 cows) Dairy Farm (mean: 0.028±0.030 µg/L; range: LOD – 0.109 µg/L) and small herd size (≤5 cows) Dairy Farm (mean: 0.029±0.089 µg/L; range: LOD – 0.513 µg/L) was not significantly different. Similarly, a highly significant level of AFM_1, 0.763±0.974 µg/L (range: 0.021 – 3.85 µg/L) in milk corresponding to the large-scale milk producing Dairy Farms than in milk samples corresponding to the medium (mean: 0.023±0.026 µg/L; range: LOD – 0.126 µg/L) and small (mean: 0.017±0.031 µg/L; range: LOD – 0.109 µg/L) scale milk producing Dairy Farms.

 doi:10.20944/preprints202403.0371.v1

Table 4. Level of AFM_1 in milk collected from Dairy Farms across herd size and milk production scales.

Categories	Mean ±SD (µg/L)	Min. (µg/L)	Max. (µg/L)	*P-value*
Herd Size				
Small scale (≤5 cows)	0.029±0.089 [a]	LOD	0.513	0.01
Medium scale (6-15)	0.028±0.030 [a]	LOD	0.109	
Large scale (≥16)	0.720±0.975 [b]	0.017	3.850	
Milk Produced/Farm (L)				
Small scale (≤20)	0.017±0.031 [a]	LOD	0.109	0.01
Medium scale (20-40)	0.023±0.026 [a]	LOD	0.126	
Large scale (≥41)	0.763±0.974 [b]	0.021	3.850	

LOD = limit of detection (≥0.008 µg/L); SD = standard deviation.

4. Discussion

Milk is widely regarded as a source of essential nutrients, that are consumed globally across different age groups, particularly, by infantry, children, pregnant women, and the elderly [20,56]. However, it could be a potential source of toxic compounds like aflatoxin M_1, which poses serious health risks, including liver cancer [57]. This study assessed the prevalence and level of AFM_1 in milk collected from indoor Dairy Farms and local Milk Vendors in three Urban Centers and compared it with the different findings from several countries (Table 5). Thus, to the best of our knowledge, a study has not been published yet, pertaining to the AFM_1 in raw milk of dairy cows from the current study areas. Therefore, in this study, aflatoxin M_1 was detected in 115/180 (63.9%) milk samples with an overall average concentration of 0.179±0.48 µg/L, which ranges from LOD (0.008 µg/L) to 3.85 µg/L. Thus, in the present study higher occurrence and level of AFM_1 in raw milk indicates that lactating cows have been exposed to concentrate feeds or feed ingredients that are prone to AFB_1 contamination [58,59], despite the determination of AFB_1 in feeds has not been the scope of this study.

Our study revealed a considerable variation in AFM_1 prevalence and concentration in milk from different regions in Ethiopia. Thus, compared to the present study, higher contamination of AFM_1 with 100% (N=110) prevalence and 4.98 µg/L average concentration in greater Addis Ababa milk sheds [27], 100% (N=108) prevalence and 0.69±0.505 µg/L average concentration in Bishoftu town [31], 100% (N=64) prevalence and 0.319±0.5 µg/L mean level in different Urban Centers of Oromia, Amhara and former SNNP region [60], and 99% (N=100) prevalence and 0.47±0.73 µg/L mean level in South Gonder Zone [61] were reported in raw milk from various locations in Ethiopia. Additionally, a higher occurrence, but relatively lower level of AFM_1 in raw milk from Addis Ababa and nearby towns (93%; average: 0.029 µg/L) and milk from different sites of Central Highland (71%; average: 0.054 µg/L) were reported in Ethiopia [28,62].

Furthermore, in some East African countries, higher occurrences of AFM_1 in raw milk of dairy cows, with a contamination rate of 95.45% (N=44; mean=2.07 µg/L) in Sudan [63], prevalence of 100% (N=96; mean: 0.290.3 µg/L) in Kenya [64] and occurrence of 83.8% (N=37) in Tanzania were reported [25]. However, consistent with this finding, the prevalence rate of AFM_1 of 68.42% (N=38), 64.2% (N=38), and 58.8% (N=701) in Yemen, Pakistan, and Lebanon were reported respectively [50,65,66]. Similarly, the average concentration of 0.183 µg/L (N=38), and 156.71 ng/L (N=84) of AFM_1 were reported in the raw milk of dairy cows of Algeria and Yemen respectively [50,67]. However, the variation in AFM_1 prevalence within different reports may be associated with different methods for toxin detection, geographical locations, agro-climatic variations, feed sources or feed ingredients, and feed storage conditions [17,48,59].

In the present study, out of the contaminated milk samples (N=115), the levels of AFM_1 in 45 (39.13%) and 30 (26.08%) samples were higher than the threshold limits of ESA/EU and FDA respectively. Compared to the current study, a higher level of AFM_1 exceeding the tolerable limit of ESA/EU (97.8%), but comparable with FDA (26.5%) was reported in the greater Addis Ababa milk

 doi:10.20944/preprints202403.0371.v1

shed in Ethiopia [27]. Likewise, 58% and 42% of milk samples from different sites of Central Highland and 96% and 82% of milk from Bishoftu town contain the concentration of AFM_1 exceeding the threshold limit of ESA/EU and FDA in Ethiopia [31,62]. However, Iqbal et al. [65] and Daou et al. [66] reported that 25% and 28% of the milk samples exceeded the tolerable limit of ESA/EU (0.05 µg/L) of AFM_1, which is relatively lower compared to the present finding.

Furthermore, data obtained in the present study indicates that Dire Dawa city, a relatively hotter Urban Center, was the origin of milk samples containing significantly higher prevalence (40.0%) as well as mean level (0.344±0.72 µg/L) of AFM_1 than in the milk samples from the Chiro town and Harar city. However, a 30.43% prevalence and 0.055±±0.13 µg/L mean level of AFM_1 in milk samples from Chiro town was not significant compared to a 29.57% prevalence and 0.140±0.33 µg/L average level in the milk samples collected from the Harar city. In agreement with the present finding, Njombwa et al., [22] reported, a significantly higher level of AFM_1 in raw milk from the hotter Lakeshore Agro-ecological Zone, compared to the Mid and Highland Agro-ecological Zones in Malawi. This notion supports that, AFB_1 is more frequently produced in the areas with high environmental temperatures and humidity, where Aspergillus fungi thrive much better than in relatively less hot and dry environments. Thus, the level of AFB_1 produced is metabolized into AFM_1 by mammals and subsequently secreted into milk [68].

Likewise, a finding in another study revealed, a considerable variation in AFM_1 contamination rate and mean level recovered from raw milk samples collected from different regions in Algeria [67]. Thus, the highest contamination of 77.27% and mean of 152.46±44.14 ng/L in milk samples from the Center North region than in the milk samples from the Northeast region (prevalence: 30.43% and mean: 32.94±11.87 ng/L) and Northwest region (prevalence: 38.64% and mean: 57.05±21.67 ng/L). In support of our finding, the authors in this study have noted that the variations of AFM_1 level or contamination in raw milk between different regions in Algeria were linked to geographical and climatic differences.

Additionally, the prevalence of 57.39% and concentration level of 0.252±0.64 µg/L of AFM_1 in milk samples collected from the specialized Dairy Farms was found significant, compared to 42.61% prevalence and 0.107±0.21 µg/L mean in the milk samples collected from the local Milk Vendors. Similar to the current study, Zebib et al., [60] reported, a higher mean concentration of AFM_1 in milk from producers/farmers (0.132 µg/L) than that of the milk from the farm gate markets (0.022 µg/L) in the former SNNP region in Ethiopia. In agreement with this finding, a study conducted in Nairobi Kenya revealed, a significantly higher level of AFM_1 in the milk samples from the Dairy Farms (627.5±238.19 µg/L) than in the samples collected from milk shops (28.8±0.0 µg/L) [64]. Similarly, the study conducted by Kirino et al., [69] concluded that AFM_1 levels in milk samples from the individual Dairy Farms were higher than in the milk samples collected from dairy shops, kiosks, vendors, and groceries.

Moreover, as shown in Table 5, the level of AFM_1 recovered from the raw milk samples among large-scale herd size (0.720±0.975 µg/L) and large-scale milk producing Dairy Farms (0.763±0.974 µg/L) were found significant compared to the Dairy Farm with medium and low-scale herd size and milk production. Similarly, despite comparable occurrence of AFM_1 in milk obtained from small (46.8%, N=47) and large (45.94%, N=37) herd size, the mean level of AFM_1 in milk samples corresponding to the large herd size Dairy Farm (90.16±43.02 ng/L) was found significantly higher than the mean level of AFM_1 recovered from the milk samples corresponding to small herd size Dairy Farm (58.59±27.44 ng/L) in Algeria [67]. This may be related that the large herd size Dairy Farms as well as large-scale milk producing Dairy Farms, the animals are mainly fed from different concentrate feeds [33], which are more prone to Aspergillus fungus and subsequent aflatoxin contamination.

Table 5. Level of AFM_1 in raw milk of dairy cows across different countries.

Countries	Methods	N	+ (%)	>EU (%)	>FDA (%)	Mean	Ref.
Ethiopia	HPLC	180	63.9	39.13	26.08	0.179±0.48 µg/L	This study

 doi:10.20944/preprints202403.0371.v1

Sudan	HPLC	44	95.45	100	83.33	2.070 µg/L	[63]
Iran	HPLC	204	80.3	56.7	-	0.660 µg/L	[70]
Egypt	HPLC	10	6	-	-	0.061 ng/L	[71]
Ethiopia	HPLC	42	93	86	-	0.029 µg/L	[28]
Yemen	HPLC	38	68.42	36.84	-	0.183 µg/L	[50]
Pakistan	HPLC	38	64.2	25	-	0.082 µg/L	[65]
Algeria	HPLC	84	46.42	-	1.19	156.71 ng/L	[67]
Lebanon	HPLC	701	58.8	28.0	-	0.035±0.051 µg/L	[66]
Kenya	HPLC	96	100	66.4	7.5	290.3±66.3 ng/L	[64]
Ethiopia	ELISA	45	71	58	42	0.054 µg/L	[62]
Ethiopia	ELISA	110	100	97.8	26.3	4.980 µg/L	[27]
Ethiopia	ELISA	108	100	96	82	0.69±0.505 µg/L	[31]
Ethiopia	ELISA	100	99	41	-	0.47±0.73 µg/L	[61]
Kenya	ELISA	72	37.5	26.4	-	-	[21]
Malawi	VICAM	112	100	98	22	0.55±0.18 µg/L	[22]
Pakistan	ELISA	340	86.66	-	34.45	0.520 µg/L	[48]
Kenya	ELISA	96	-	-	-	627.5±238.19 ng/L	[64]
Kenya	ELISA	150	100	58	-	-	[20]
Kenya	ELISA	84	98.8	64	-	83.66±64.68 ng/L	[19]
Pakistan	HPLC	28	64.2	25	-	82.4 ± 7.8 ng/L	[65]
Algeria	ELISA	84	46.42	-	1.19	71.92±28.48 ng/L	[67]
Iran	ELISA	180	77.2	22.7	-	56.32 ± 74.37 ng/L	[56]
Sudan	VICAM	25	92	-	-	-	[23]
Rwanda	VICAM	170	-	91.8	38.8	0.89±1.64 µg/L	[24]
Tanzania	HPLC	-	30.7	27.9	-	-	[26]
Tanzania	HPLC	37	83.8	100	16.1	-	[25]

5. Conclusions

Aflatoxin M_1 was found, highly prevalent in the raw milk of dairy cows collected from the selected Urban Centers in Eastern Ethiopia, with an overall prevalence of 63.9% and an average of 0.179±0.48 µg/L. The AFM_1 level in 45 (39.13%) and 30 (26.08%) milk samples were higher than the thresholds set by ESA/EU and FDA respectively. The prevalence as well as average concentration of AFM_1 in the milk samples was found significant across the Urban Centers and Milk Sources. Thus, a statistically higher prevalence and level of AFM_1 in milk collected from Dire Dawa city were found, compared to the other Urban Centers. Similarly, higher contamination and levels of AFM_1 in milk samples collected from Dairy Farms than the samples collected from Milk Vendors were observed in this study. Therefore, urgent interventions, such as milk quality monitoring and regulatory measures, together with the awareness creation among the different dairy stakeholders are very critical to mitigate AFM_1 contamination in milk and milk products in study locations, especially, where higher rate of contamination has been observed. Moreover, further studies, on the factors contributing to aflatoxin contamination in cow milk, particularly, the interaction of seasonal weather variation with aflatoxin in milk is commendable.

Acknowledgments: The findings highlighted in this article were able to be produced with the support of several steamed individuals, community members, institutions, government sectors, and others. Therefore, the authors would like to acknowledge those parties directly or indirectly contributed to the completion of this research.

Further, the authors would like to acknowledge the FDRE Ministry of Education, Haramaya University, and Bule Hora University for partial support of the research activities and payment of the salary during the study period.

Conflicts of Interest: The authors declared that there is no conflict of interest between the authors and organizations regarding this paper.

References

1. Chhaya, R.S.; O'Brien, J.; Cummins, E. Feed to Fork Risk Assessment of Mycotoxins under Climate Change Influences - Recent Developments. *Trends Food Sci. Technol.* **2022**, *126 (2022)*, 126–141. https://doi.org/10.1016/j.tifs.2021.07.040.
2. Daou, R.; Joubrane, K.; Maroun, R.G.; Khabbaz, L.R.; Ismail, A.; El Khoury, A. Mycotoxins: Factors Influencing Production and Control Strategies. *AIMS Agric. Food* **2021**, *6* (1), 416–447. https://doi.org/10.3934/AGRFOOD.2021025.
3. Alameri, M.M.; Kong, A.S.Y.; Aljaafari, M.N.; Ali, H. Al; Eid, K.; Sallagi, M. Al; Cheng, W.H.; Abushelaibi, A.; Lim, S.H.E.; Loh, J.Y.; Lai, K.S. Aflatoxin Contamination: An Overview on Health Issues, Detection and Management Strategies. *Toxins (Basel).* **2023**, *15* (4), 1–16. https://doi.org/10.3390/toxins15040246.
4. Klingelhofer, D.; Zhu, Y.; Braun, M.; Bendels, M.H.K.; Brüggmann, D.; Groneberg, D.A.; Klingelhöfer, D.; Zhu, Y.; Braun, M.; Bendels, M.H.K.; Brüggmann, D.; Groneberg, D.A. Aflatoxin – Publication Analysis of a Global Health Threat. *Food Control* **2018**, *89* (2018), 280–290. https://doi.org/10.1016/j.foodcont.2018.02.017.
5. Ramesh, J.; Sarathchandra, G.; Sureshkumar, V. Analysis of Feed Samples for Aflatoxin B1 Contamination by HPTLC: A Validated Method. *Int. J. Curr. Microbiol. App. Sci.* **2013**, *2* (5), 373–377.
6. Iqbal, S.Z.; Jinap, S.; Pirouz, A.A.; Ahmad Faizal, A.R. Aflatoxin M1 in Milk and Dairy Products, Occurrence and Recent Challenges: A Review. *Trends Food Sci. Technol.* **2015**, *46* (1), 110–119. https://doi.org/10.1016/j.tifs.2015.08.005.
7. FAO. Agricultural Production Statistics 2000–2021. *Faostat Analytical Brief 60*. Food and Agriculture Organization of the United Nations Rome, Italy 2022. https://doi.org/10.4060/cc3751en.
8. Hassouna, K. Ben; Salah-Abbès, J. Ben; Chaieb, K.; Abbès, S.; Ferrer, E.; Martí-Quijal, F.J.; Pallarés, N.; Berrada, H. The Occurrence and Health Risk Assessment of Aflatoxin M1 in Raw Cow Milk Collected from Tunisia during a Hot Lactating Season. *Toxins (Basel).* **2023**, *15* (9), 1–13. https://doi.org/10.3390/toxins15090518.
9. CSA. *Federal Democratic Republic of Ethiopia Central Statistical Agency Agricultural Sample Survey 2020/21 [2013 E.C.] Volume II Report on Livestock and Livestock Characteristics (Private Peasant Holdings)*; Addis Ababa, Ethiopia, 2021; Vol. 589.
10. Valencia-Quintana, R.; Milić, M.; Jakšić, D.; Klarić, M.Š.; Tenorio-Arvide, M.G.; Pérez-Flores, G.A.; Bonassi, S.; Sánchez-Alarcón, J. Environment Changes, Aflatoxins, and Health Issues, a Review. *Int. J. Environ. Res. Public Health* **2020**, *17* (21), 1–10. https://doi.org/10.3390/ijerph17217850.
11. IARC. Mycotoxins and Human Health. *IARC Sci. Publ.* **2012**, No. 158, 87–104.
12. Antunes, I.C.; Bexiga, R.; Pinto, C.; Roseiro, L.C.; Quaresma, M.A.G. Cow's Milk in Human Nutrition and the Emergence of Plant-Based Milk Alternatives. *foods* **2023**, *12* (99), 1–21.
13. Awuchi, C.G.; Nyakundi Ondari, E.; Josiah Eseoghene, I.; Twinomuhwezi, H.; Otuosorochi Amagwula, I.; Morya, S.; Ondari, E.N.; Eseoghene, I.J.; Twinomuhwezi, H.; Amagwula, I.O.; Morya, S.; Nyakundi Ondari, E.; Josiah Eseoghene, I.; Twinomuhwezi, H.; Otuosorochi Amagwula, I.; Morya, S. Fungal Growth and Mycotoxins Production: Types, Toxicities, Control Strategies, and Detoxification. *Fungal Reprod. Growth* **2022**, No. September, 1–20. https://doi.org/10.5772/intechopen.100207.
14. Kumar, P.; Mahato, D.K.; Kamle, M.; Mohanta, T.K.; Kang, S.G. Aflatoxins: A Global Concern for Food Safety, Human Health and Their Management. *Front. Microbiol.* **2017**, *7* (2170), 1–10. https://doi.org/10.3389/fmicb.2016.02170.
15. Medina, A.; Rodriguez, A.; Magan, N. Effect of Climate Change on Aspergillus Flavus and Aflatoxin B1 Production. *Front. Microbiol.* **2014**, *5* (JULY), 1–7. https://doi.org/10.3389/fmicb.2014.00348.
16. Hell, K.; Mutegi, C. Aflatoxin Control and Prevention Strategies in Key Crops of Sub-Saharan Africa. *African J. Microbiol. Res.* **2011**, *5* (5), 459–466.
17. Tadele, F.; Demissie, B.; Amsalu, A.; Demelash, H.; Mengist, Z.; Ambelu, A.; Yenew, C. Aflatoxin Contamination of Animal Feeds and Its Predictors among Dairy Farms in Northwest Ethiopia: One Health Approach Implications. *Front. Vet. Sci.* **2023**, *10* (5). https://doi.org/10.3389/fvets.2023.1123573.
18. Benkerroum, N. Aflatoxins: Producing-molds, Structure, Health Issues and Incidence in Southeast Asian and Sub-saharan African Countries. *Int. J. Environ. Res. Public Health* **2020**, *17* (4). https://doi.org/10.3390/ijerph17041215.

19. Kagera, I.; Kahenya, P.; Mutua, F.; Anyango, G.; Kyallo, F.; Grace, D.; Lindahl, J. Status of Aflatoxin Contamination in Cow Milk Produced in Smallholder Dairy Farms in Urban and Peri-Urban Areas of Nairobi County: A Case Study of Kasarani Sub County, Kenya. *Infect. Ecol. Epidemiol.* **2019**, *9* (1), 1547095. https://doi.org/10.1080/20008686.2018.1547095.
20. Langat, G.; Tetsuhiro, M.; Gonoi, T.; Matiru, V.; Bii, C. Aflatoxin M1 Contamination of Milk and Its Products in Bomet County, Kenya. *Adv. Microbiol.* **2016**, *06*, 528–536. https://doi.org/10.4236/aim.2016.67053.
21. Anyango, G.; Mutua, F.; Kagera, I.; Andang'O, P.; Grace, D.; Lindahl, J.F. A Survey of Aflatoxin M1 Contamination in Raw Milk Produced in Urban and Peri-Urban Areas of Kisumu County, Kenya. *Infect. Ecol. Epidemiol.* **2018**, *8* (1), 1–11. https://doi.org/10.1080/20008686.2018.1547094.
22. Njombwa, C.A.; Moreira, V.; Williams, C.; Aryana, K.; Matumba, L. Aflatoxin M1 in Raw Cow Milk and Associated Hepatocellular Carcinoma Risk among Dairy Farming Households in Malawi. *Mycotoxin Res.* **2020**, *37* (1), 89–96. https://doi.org/10.1007/s12550-020-00417-5.
23. Fadlalla, A.A.; El-Zubeir, I.E.M.; Elnahas, A. Aflatoxin M1 Contamination in Fluid Milk Products, in Khartoum State, Sudan. *Vet. Med. Public Heal. J.* **2020**, *1* (2), 34–40. https://doi.org/10.31559/vmph2020.1.2.2.
24. Nishimwe, K.; Bowers, E.L.; Ayabagabo, J.D.; Habimana, R.; Mutiga, S.; Maier, D.E. Preliminary Sampling of Aflatoxin M1 Contamination in Raw Milk from Dairy Farms Using Feed Ingredients from Rwanda. *Mycotoxin Res.* **2022**, *38* (2), 107–115. https://doi.org/10.1007/s12550-022-00454-2.
25. Mohammed, S.; Munissi, J.J.E.; Nyandoro, S.S. Aflatoxin M1 in Raw Milk and Aflatoxin B1 in Feed from Household Cows in Singida, Tanzania. *Food Addit. Contam. Part B Surveill.* **2016**, *9* (2), 85–90. https://doi.org/10.1080/19393210.2015.1137361.
26. Kitigwa, S.J.; Kimaro, E.G.; Nagagi, Y.P.; Kussaga, J.B.; Suleiman, R.A.; Matemu, A. Occurrence and Associated Risk Factors of Aflatoxin Contamination in Animal Feeds and Raw Milk from Three Agroecological Zones of Tanzania. *World Mycotoxin J.* **2023**, *16* (2), 149–163. https://doi.org/10.3920/WMJ2022.2801.
27. Dawit, G.; Szonyi, B.; Azage, T.; Hanson, J.; Grace, D.; Gizachew, D.; Szonyi, B.; Tegegne, A.; Hanson, J.; Grace, D. Aflatoxin Contamination of Milk and Dairy Feeds in the Greater Addis Ababa Milk Shed, Ethiopia. *Food Control* **2016**, *59 (2016)*, 773–779. https://doi.org/10.1016/j.foodcont.2015.06.060.
28. Abenet, W. Comparative Study of Aflatoxin M1 in Milk Samples Marketed in Capital City and Rural Place, MSc Thesis. Department of Food and Nutritional Sciences, Addis Ababa University, Addis Ababa Ethiopia., 2017.
29. Yohannes, B.; Ayalew, W.; Getachew, A. Analysis to Ascertain the Determination for Aflatoxin Contamination of Milk and Feeds from Gurage Zone, Ethiopia. *J. Agric. Res.*, **2018**, *13* (1), 1–11. https://doi.org/10.3923/ijar.2018.1.11.
30. Zebib, H.; Abate, D.; Woldegiorgis, A.Z. Aflatoxin M1 in Raw Milk, Pasteurized Milk and Cottage Cheese Collected along Value Chain Actors from Three Regions of Ethiopia. *Toxins (Basel).* **2022**, *14* (276), 1–11.
31. Tadesse, S.; Berhanu, T.; Zewdu, A. Aflatoxin M1 in Milk and Milk Products Marketed by Local and Industrial Producers in Bishoftu Town of Ethiopia. *Food Control* **2020**, *118* (107386), 1–10. https://doi.org/10.1016/j.foodcont.2020.107386.
32. CSA. Population Projections for Ethiopia 2007-2037. *Central Statistical Agency*. Federal Democratic Republic of Ethiopia, Central Statistical Ageny: Addis Ababa, Ethiopia 2013, p 188.
33. Alemu, M.M. Urban and Peri-Urban Dairy Cattle Production in Ethiopia: A Review. *Online J. Anim. Feed Res.* **2019**, *9* (4), 173–177.
34. Shapiro, B.I.; Gebru, G.; Desta, S.; Negassa, A.; Negussie, K.; Aboset, G.; Mecha, H. *Ethiopia Livestock Master Plan. ILRI Project Report. Nairobi, Kenya: International Livestock Research Institute (ILRI).*; Nairobi, Kenya, 2020.
35. Tegegn, A.; Baudronb, F.; Wegary, D. Comparative Performance of Five Maize Varieties as Livestock Feed in the Rift Valley of Ethiopia. *Acad. Res. J. Agric. Sci. Res.* **2017**, *5* (5), 366–379. https://doi.org/10.23880/oajar-16000143.
36. Teshome, D.; Fita, L.; Feyissa, F.; Kitaw, G.; Wondatir, Z. Effect of Total Mixed Ration on Dry Matter Intake, Milk Yield and Composition Effect of Total Mixed Ration on Dry Matter Intake, Milk Yield and Composition of Early Lactating Jersey Cows. *J. Biol. Agric. Healthc.* **2019**, *7* (January), 19–24.
37. Zeleke, M. Ethiopia's Livestock Systems: Overview and Areas of Inquiry. Feed the Future Innovation Lab for Livestock Systems. Gainesville, FL, USA 2021, p 54. https://doi.org/10.4324/9781003319665-11.
38. Ahmedin, A.; Yesihak, Y. Milk Production Performance, Challenges and Opportunities of Dairy Cattle Production in West Hararghe, Oromiya Regional State. *Open J. Anim. Sci.* **2020**, *10* (01), 219–235. https://doi.org/10.4236/ojas.2020.101012.
39. Brandsma, W.; Mengistu, D.; Kassa, B.; Yohannes, M.; Lee, J. van der. The Major Ethiopian Milksheds; Wageningen, Wageningen UR (University & Research Centre) Livestock Research. Livestock Research Report 735, 245 blz, December Lelystad, Netherlands 2012. https://doi.org/10.13140/RG.2.1.1354.2246.

 doi:10.20944/preprints202403.0371.v1

40. Mohammed, Y.K.; Waal, H.O. de. Herd Management, Milk Production and Reproduction of Urban Dairy Farms in the Harar Milk Shed. *Ethiop. J. Anim. Prod.* **2009**, *9* (1), 57–75.
41. Lemma, S.; Dagne, T.; Gashaw, G.; Getu, A. Assessment of Cow's Milk Hygienic Practices Under Small Scale Farmers in West Hararghe Zone, Oromia National Regional State, Ethiopia. *Adv. Life Sci. Technol.* **2018**, *68* (2018), 46–55.
42. Mengistu, K.; Mohammed, A.; Eyassu, S.; Tarekegn, G.; Estifanos, H.; Yonas, H. The Dairy Value Chain and Factors Affecting Choice of Milk Channels in Harar and Dire Dawa Areas, Eastern Ethiopia. *Rev. Agric. Appl. Econ.* **2016**, *19* (2), 10–18. https://doi.org/10.15414/raae/2016.19.02.10-18.
43. Kebede, A.; Balina, A.; Kebede, A.; Tamiru, Y. Review on Aflatoxin and Its Impacts on Livestock. *J. Dairy Vet. Sci.* **2018**, *6* (2), 1–7. https://doi.org/10.19080/JDVS.2018.06.555685.
44. Abibeker, S.A.; Mume, A.A.; Mariye, M.; Desalegn, D.G.; Furgasa, W. Causes of Water Pollution in Chiro River Eastern Oromia, Ethiopia. *Int. J. Sci. Res. Publ.* **2023**, *13* (7), 111–119. https://doi.org/10.29322/IJSRP.13.07.2023.p13912.
45. Arabali, M.; Amare, E.G. A Cross Sectional Study on Prevalence of Cephalopina Titillator Infection in Camel (Camelus Dromedaries) in Dire Dawa Administrative Region, Ethiopia. *Adv. Biol. Res. (Rennes).* **2015**, *9* (4), 225–229. https://doi.org/10.5829/idosi.abr.2015.9.4.94209.
46. Biri, A.; Ketema, K.; Ayele, S.; Lule, D. Analysis of Crop Production Constraints Through Participatory Rural Appraisal in Harari Region, Eastern Ethiopia; Implications for Research and Development. *J. Agric. Crop.* **2019**, *5* (10), 209–217.
47. Daniel, W.W.; Cross, C.L. *Biostatistics: A Foundation for Analysis in the Health Sciences.*, 10th ed.; Daniel, W.W and Cross, C. L., Ed.; New York: John Wiley and Sons, 2013. https://doi.org/10.2307/2531929.
48. Tahira, I.; Sultana, N.; Munir, A.; Hasan, S.M.; Hanif, N.Q. Occurrence of Aflatoxin M1 in Raw and Processed Milk Consumed in Pakistan. *Pak. J. Pharm. Sci.* **2019**, *32* (3), 1097–1101.
49. AOAC. Official Methods of Analysis of the Association of Analytical Chemists. *AOAC Official Method Washington DC, 935.14 and 992.24.* 2005, p 334. https://doi.org/10.1002/jps.2600600253.
50. Murshed, S. Evaluation and Assessment of Aflatoxin M1 in Milk and Milk Products in Yemen Using High-Performance Liquid Chromatography. *J. Food Qual.* **2020**, *2020*, 8. https://doi.org/10.1155/2020/8839060.
51. EC. *European Commission (EU) Document Nº SANTE/2015/11945. Guidance document on analyttical quality control and method validation procedures for pesticides residue analysis in food and feeds.* https://www.eurl-pesticides.eu/library/docs/allcrl/%0AAqcGuidance_SANTE_2015_11945.pdf.
52. Kassa, A.; Talema, A.; Ketsela, G. DETERMINATION OF AFLATOXIN M1 IN RAW COW'S MILK BY USING HPLC- FLD, IN INJIBARA TOWN, AWI ZONE, AMHARA, ETHIOPIA, MSc Thesis, Department of Chemistry, College of Natural and Computational Sciences, Injibara University, Injibara, Ethiopia, 2020. https://doi.org/10.20944/preprints202010.0181.v1.
53. ESA. *Unprocessed Whole/Raw Cow Milk Specification. Ethiopian Standard Agency. 2nd Ed., ES: 3460*; 2009.
54. EU. Commission Regulation (EC) No. 165/2010 of 26 February 2010 Amending Regulation (EC) No. 1881/2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs as Regards Aflatoxins. *Off. J. Eur. Union* **2010**, *50*, 8–12.
55. FDA. *Whole Milk, Lowfat Milk, Skim Milk - Aflatoxin M1. FDA Available online:* https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cpg-sec-527400-whole-milk-lowfat-milk-skim-milk-aflatoxin-m1 (accessed on 29 February 2024).
56. Rahimzadeh Barzoki, H.; Faraji, H.; Beirami, S.; Keramati, F.Z.; Nayik, G.A.; Izadi Yazdanaabadi, Z.; Mozaffari Nejad, A.S. Seasonal Study of Aflatoxin M1 Contamination in Cow Milk on the Retail Dairy Market in Gorgan, Iran. *Dairy* **2023**, *4* (4), 571–580. https://doi.org/10.3390/dairy4040039.
57. Hashemi, M. A Survey of Aflatoxin M1 in Cow Milk in Southern Iran. *J. Food Drug Anal.* **2016**, *24* (4), 888–893. https://doi.org/10.1016/j.jfda.2016.05.002.
58. Sahin, H.Z.; Celik, M.; Kotay, S.; Kabak, B. Aflatoxins in Dairy Cow Feed, Raw Milk and Milk Products from Turkey. *Food Addit. Contam. Part B Surveill.* **2016**, *9* (2), 152–158. https://doi.org/10.1080/19393210.2016.1152599.
59. Robert, O.A.; Nyamete, F.A.; Kabululu, M.L.; Ngungulu, A.W. Aflatoxins Contamination in Animal Feeds and Fresh Milk in Kondoa District, Tanzania. *Eur. J. Nutr. Food Saf.* **2023**, *15* (12), 10–21. https://doi.org/10.9734/ejnfs/2023/v15i121364.
60. Zebib, H.; Abate, D.; Woldegiorgis, A.Z. Exposure and Health Risk Assessment of Aflatoxin M1 in Raw Milk and Cottage Cheese in Adults in Ethiopia. *Foods* **2023**, *12* (4), 1–11. https://doi.org/10.3390/foods12040817.
61. Admasu, F.T.; Melak, A.; Demissie, B.; Yenew, C.; Legesse, M.; Tefera, T.; Teka, O.; Ermias, S.; Tiruneh, G.M.; Malik, T.; Dejenie, T.A.; Habtie, M.L.; Bekele, T.T.; Feyesa, T.O.; Chanie, E.S.; G/Medhin, M.T.; Malik, T.; Dejenie, T.A. Occurrence and Associated Factors of Aflatoxin M1 in Raw Cow Milk in South Gondar

 doi:10.20944/preprints202403.0371.v1

Zone, North West Ethiopia. *Food Sci. Nutr.* **2021**, *9* (11), 6286–6293. https://doi.org/10.1002/fsn3.2589.
62. Rehrahie, M.; Getnet, A.; Fassil, A. Determination of Aflatoxin in Dairy Feeds and Milk in Some Selected Areas of Ethiopia. *Food Environ. Saf. -* **2018**, *XVII* (3), 286–299.
63. Elzupir, A.O.; Elhussein, A.M. Determination of Aflatoxin M1 in Dairy Cattle Milk in Khartoum State, Sudan. *Food Control* **2010**, *21* (6), 945–946. https://doi.org/10.1016/j.foodcont.2009.11.013.
64. Kuboka, M.M.; Imungi, J.K.; Njue, L.; Mutua, F.; Grace, D.; Lindahl, J.F. Occurrence of Aflatoxin M1 in Raw Milk Traded in Peri-Urban Nairobi, and the Effect of Boiling and Fermentation. *Infect. Ecol. Epidemiol.* **2019**, *9* (1), 1–8. https://doi.org/10.1080/20008686.2019.1625703.
65. Iqbal, S.Z.; Waqas, M.; Latif, S. Incidence of Aflatoxin M1 in Milk and Milk Products from Punjab, Pakistan, and Estimation of Dietary Intake. *Dairy* **2022**, *3* (3), 577–586. https://doi.org/10.3390/dairy3030041.
66. Daou, R.; Afif, C.; Joubrane, K.; Khabbaz, L.R.; Maroun, R.; Ismail, A.; Khoury, A. El. Occurrence of Aflatoxin M1 in Raw, Pasteurized, UHT Cows' Milk, and Dairy Products in Lebanon. *Food Control* **2020**, *111* (107055), 1–29. https://doi.org/10.1016/j.foodcont.2019.107055.
67. Mohammedi-Ameur, S.; Dahmane, M.; Brera, C.; Kardjadj, M.; Ben-Mahdi, M.H. Occurrence and Seasonal Variation of Aflatoxin M1 in Raw Cow Milk Collected from Different Regions of Algeria. *Vet. World* **2020**, *13* (3), 433–439. https://doi.org/10.14202/vetworld.2020.433-439.
68. Li, H.; Xing, L.; Zhang, M.; Wang, J.; Zheng, N. The Toxic Effects of Aflatoxin B1 and Aflatoxin M1 on Kidney through Regulating L-Proline and Downstream Apoptosis. *Biomed Res. Int.* **2018**, *2018*, 1–11. https://doi.org/10.1155/2018/9074861.
69. Kirino, Y.; Makita, K.; Grace, D.; Lindahl, J. Survey of Informal Milk Retailers in Nairobi, Kenya and Prevalence of Aflatoxin M1 in Marketed Milk. *African J. food, Agric. Nutr. Dev.* **2016**, *03* (Special), 11022–11033. https://doi.org/10.3143/geriatrics1960.1963.Special_120.
70. Fallah, A.A.; Barani, A.; Nasiri, Z. Aflatoxin M1 in Raw Milk in Qazvin Province, Iran: A Seasonal Study. *Food Addit. Contam. Part B Surveill.* **2015**, *8* (3), 195–198. https://doi.org/10.1080/19393210.2015.1046193.
71. Younis, G.; Ibrahim, D.; Awad, A.; El Bardisy, M.M. Determination of Aflatoxin M1 and Ochratoxin A in Milk and Dairy Products in Supermarkets Located in Mansoura City, Egypt. *Adv. Anim. Vet. Sci.* **2016**, *4* (4), 114–121. https://doi.org/10.14737/JOURNAL.AAVS/2016/4.2.114.121.