Aflatoxin contamination in cow milk during wet and dry season in selected rural areas of Sidama zone Southern Ethiopia

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ABSTRACT
Aflatoxin M₁ was determined in 240 milk samples collected from Hulla and Dale districts, Ethiopia, by Enzyme linked immunosorbent assays (ELISA) detection. It has been found that 96.7% of the respondents did not have any awareness about aflatoxin. Laboratory analysis showed that AFM₁ was detected in 77.91% of the total raw milk samples collected within a range of 0.00–0.038 and with an average of 0.0068 µg/L. Average AFM₁ in raw milk during dry season (0.008 ± 0.0003 µg/L) was significantly higher (P < 0.05) than wet season (0.0049 ± 0.0041 µg/L). The level AFM₁ detected in raw milk under the current study is lower than the limits set by EU and USA and hence can be considered as safe milk to consume. But the lower level of awareness about aflatoxin in the farmers showed that there is risk of aflatoxin contamination, so awareness creation and aflatoxin control measured should be implemented.

Introduction
Mycotoxins are fungal secondary metabolites when ingested cause a variety of adverse effects in both humans and animals.[1] Aflatoxins are the most toxic class of mycotoxins produced mainly by Aspergillus flavus and A. parasiticus.[2] These fungal metabolites are primarily found in various plants and their grains, such as nuts and maize. Aspergillus flavus and A. parasiticus especially represent 93% of strains that produce aflatoxins worldwide.[3,4] There are more than 20 known aflatoxins that have been identified. The four main ones are aflatoxin B₁ (AFB₁), aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁), and aflatoxin G₂ (AFG₂).[5] Aflatoxin M₁ (AFM₁) and M₂ (AFM₂) are the hydroxylated metabolites of AFB₁ and AFB₂.[6] Letter “B” and “G” refer to the blue and green fluorescent colors produced under UV light on thin layer chromatography plates, while the subscript numbers 1 and 2 indicate major and minor compounds, respectively.[3]

Aflatoxins have an adverse effects in humans, animals, and on crops.[7,8] When ingested they are converted into toxic compounds that can have an adverse effect on the liver and may cause hepatic cancer and acute liver cirrhosis[9,10] as well as damage to lungs, kidneys, brains and heart as evidenced from dead individuals with high levels of aflatoxin contamination. In humans and many animals, its higher consumptions cause acute toxicity including death. However, in terms of food safety chronic toxicity is considered as a more significant risk.[8] The association between chronic aflatoxicosis and liver cancer has been reported.[11] In addition, AFM₁ is classified by the International Agency for Research on Cancer (IARC) as class I carcinogens.[12]

Animal products including milk can also be affected because of the animal exposure to contaminated feed. Human exposure results directly from consumption of plant based toxins or indirectly
through the toxins/metabolites in animal products.\textsuperscript{[13]} The US Food and Drug Administration (FDA) considers aflatoxin as an unavoidable contaminant and the goal has been to detect and minimize contamination of food.\textsuperscript{[11]} Aflatoxin is a major risk factor of liver cancer, which can be as – the sixth leading cause of cancer deaths worldwide. Globally, liver cancer causes more than 700,000 new cases per annum (World Cancer Research Fund International, 2015), of which up to a quarter can be attributable to Aflatoxin exposure.\textsuperscript{[9]}

Ethiopia is one of the leading countries by livestock population from Africa and has emerging dairy industry. Milk produced from rural areas account 97\% of the total national milk production and 75\% of commercialized milk production. Out of the total milk produced in the rural area 85\% is used for household consumptions.\textsuperscript{[14]} The rural milk production is characterized by indigenous breed and the common feed types are natural grass hay, crop residues (straws and chaffs of cereals and pulps), and free grazing grass which are difficult to monitoring.\textsuperscript{[14]}

Therefore, people in the country have high possibility of exposing to AFM\textsubscript{1}, since the use of cow raw milk and its products are common practice in the dairy farmers. However, limited evidence is available regarding to the extent of aflatoxin contamination and exposure of feeds, crops and farm animals and human. A recent study Addis Ababa milk shed found high levels of AFM\textsubscript{1} in raw cow’s milk and AFB\textsubscript{1} in dairy feed from EU and USA limit.\textsuperscript{[15]} This has scared consumers and has affected the market of both milk producers and processors. Smallholder dairy farmers, whose daily lives depend on the sale of milk and other dairy products, are hard hit by the fall in demand. Studies conducted by\textsuperscript{[16]} in the Bishoftu, Sululta and Debirebirhan site indicated that 93\% of the tested samples were contaminated with AFM1. Another study reported by\textsuperscript{[17]} in the central highlands of Ethiopia also exhibited that 71\% of the tested milk samples collected from three study sites Bishoftu, Holeta and Hawassa tested positive for aflatoxin. However, major portion of milk is produced in rural areas of the country and limited published data are available about the level of aflatoxin in the cow milk, studying aflatoxin contamination is important to increase the confidence of the consumers and use it as a base line information for policy development at country level. Therefore, this study was aimed to evaluate awareness about aflatoxin and to determine level of aflatoxin M\textsubscript{1} contamination in raw cow milk.

**Materials and methods**

**Study areas**

This study was conducted in Dale and Hula districts of Sidama zone, Southern Nation Nationalities and People Regional States (SNNPR) of Ethiopia. These districts were purposely selected to represent rural area of Sidama zones. The study involve a cross-sectional survey aimed to assess awareness about aflatoxin and a laboratory-based study employed to determine occurrence and level of aflatoxin in the two seasons wet (July 2017) and dry (March 2018).

**Sample size and Sampling approach**

The minimum sample size was determined according to\textsuperscript{[18]}

\[
n = \frac{z^2P(1 - P)}{\delta^2}
\]

(1)

where \(n\) is minimum sample size, \(z\) is standard normal deviate that corresponds to 95\% confidence interval (1.96), \(P\) is estimated prevalence, \(\delta\) is the level of significance (5\%). Since there is little information on the occurrence of AFM\textsubscript{1} in cow milk in Ethiopia rural area. Prevalence rate of 80\% was used to calculate the sample size. Assuming this prevalence at 95\% confidence interval the minimum estimated sample size was given as 245.8 approximately 240 samples were taken using the formula given below. Therefore:

\[
n = (1.96)^2 \times 0.8(1 - 0.8) = 245.8(0.05)^2
\]

(2)
Initially, the total sample size (n = 120) of one season was equally distributed to the two districts (60 per district). Then, two peasant associations were selected randomly in each of the study districts. From each peasant association 30 subjects that fulfill the inclusion criteria (households owning at least one lactating cow) were also selected randomly. Prior to the data collection households that meet the inclusion criteria were listed through rapid enumeration and the list was ultimately used as a sampling frame. Similar sampling procedure was followed during the dry season.

Data collection

Questionnaire-based data collection: The socio-demographic characteristics of the respondents and knowledge and practice of the selected households in relation to Aflatoxin were assessed using structured and semi-structured questionnaire. Respondents were the household heads and mothers.

Raw milk sample collection: From thoroughly mixed raw milk, approximately 40 ml samples were collected from individual household by sterile falcon tubes. The sample tubes were labeled with identification number. The samples were collected and transported to the food Science and technology laboratory of the School of Human Nutrition, Food Science and Technology in an icebox. The samples were frozen and stored at –20°C until analysis.

Laboratory analysis

Analysis of AFM₁ in milk: Aflatoxin M1 level was determined using enzyme-linked immunosorbent assay (ELISA) kit and reagent supplied by Helica (Helica: Santa Ana, CA, USA, 2016). The limit of detection was 5 pg/ml (part per trillion [ppt]). All the tests were performed in duplicate. Milk fat was removed by centrifuging the milk samples for 5 min at 4000 g. After removing the upper cream layer by aspiration, the lower phases were used for quantitative testing. An assay protocol (#961AFLM01M-96) provided by the manufacturer was followed for determining the AFM₁[^19].

Data analysis

The data were analyzed using SPSS V.20 software. A sample was considered as positive when the result was above the limit of detection. A value of zero was assigned to the samples that presented aflatoxin concentration values lower than the limit of detection (5 pg/ml). Aflatoxin contaminations in different samples were described using the appropriate measure of central tendency and dispersion. Independent t-test was conducted to determine differences in AFM₁ concentrations in cow milk samples between wet and dry season. A P-value less than 0.05 was used to determine the level of statistical significance among the treatments of the study. Concentrations of AFM₁ were expressed as Mean ± SD, minimum and maximum value. Two-way ANOVA was performed for season – location interaction effect of AFM₁ using the model below.

\[
Y_{ij} = \mu + A_i + B_j + A_iB_j + e_{ij}
\]

where \(Y_{ij}\) is observation \(i^{th}\) location and \(j^{th}\), \(\mu\) is over all mean \(A_i\) is the effect of location \(1\) Location \(1 = \text{Dale}\) \(2 = \text{Hula}\), \(B_j\) is the effect of season \(j\), Season \(1\) is wet, \(2\) is dry, \(A_iB_j\) is interaction, \(E_{ij}\) is experimental error.

Example,
Aflatoxin contamination in Dale and hula site = over all mean + AFM₁ in Dale location + AFM₁ Dry season + AFM₁ in Dale location* AFM₁ Dry season + Experimental error
Results and discussion

Socio-demographic characteristics of respondent

Majority of the respondents (92%) in the study area were female (Table 1). This might be an indicator that females are participating more in dairy cow management like cleaning of barn, milking and feeding of the animals. The result of this study is in contrast with the finding reported by[20] that most of the households sampled for the study were male (77.5–97.4%).[21] Besides another study also revealed as majority (80%) of the respondents were male. Most of the respondents were married under the age category of 18–33 years. This indicated that most of the dairy cow husbandry practices were headed by productive and adult age group of the society which is similar with a study reported by[21] who stated that most of the dairy cow management was headed by adult and older group of the society.

Education is one of the key factors for the development of agriculture in the country side. Educated people can easily understand and accept new ideas that can transform their livelihoods. Majority of the respondents (69.2%) of the current study were educated which varied from primary to higher education. The results indicate the possibility of creating awareness using different intervention options like training, using media access and social activities easily. Only 30.8% were illiterate which is lower than the 60% illustrate respondents reported by[21] in south west Shewa zone of Oromia Region, Ethiopia. In the current study, respondents of Hula and Dale districts were not significantly difference (P > .05) in terms of their awareness about AFM1. In the study area, majority of households earn their living from agriculture which is agreed with the report of[22] in Dale and Shebedino districts where 79% and 99% of the respondents were engaged in agricultural activities, respectively.

Knowledge attitude and practice of household dairy farmers

In the study area, (96.7%) of the respondent do not have any knowledge about aflatoxin (Table 2). Similar results have been revealed by[16] in Bishoftu, Sululta and Debrebirhan district dairy farmers where 90% of the respondent did not have any knowledge about aflatoxin. Another study by[23] reported that almost all farmers (98.7%) and traders (96.7%) were unaware of aflatoxin and its consequences. Majority of the respondents did not treat the moldy feed (61.7%) while the rest treat the feed by drying in the sun. Out of all, 75.8% of the respondents throwaway moldy feed and food leftover. This is good practice to prevent Mold formation on feed because food and feed leftover have high moisture that encourages Mold growth and mycotoxin formation. The rest gave the moldy feed and food leftover for their cows (8.3%) and chicken (14.2%).

Table 1. Socio-demographic characteristics of household dairy farmers in the study site.

| Parameter          | Categories       | Dale (N = 120) | Hula (N = 120) | Total (N = 240) |
|--------------------|------------------|----------------|---------------|-----------------|
|                    |                  | %              | %             |                 |
| **Sex**            |                  |                |               |                 |
| Male               |                  | 10             | 36.6          | 23.3            |
| Female             |                  | 90             | 63.3          | 76.6            |
| **Age**            |                  |                |               |                 |
| 18–33              |                  | 90             | 88.3          | 89.1            |
| 34–41              |                  | 6.6            | 6.6           | 6.6             |
| >41                |                  | 3.3            | 5             | 4.16            |
| **Marital status of respondent** |                  |                |               |                 |
| Single             |                  | -              | 8.33          | 4.16            |
| Married            |                  | 100            | 90            | 95              |
| Divorced           |                  | -              | 1.6           | 0.83            |
| **Educational**    |                  |                |               |                 |
| Illiterate         |                  | 20             | 41.6          | 30.8            |
| Read and write     |                  | 10             | 8.3           | 9.1             |
| Primary school     |                  | 50             | 33.3          | 41.6            |
| Secondary school   |                  | 18.3           | 16.6          | 17.5            |
| College            |                  | 1.6            | -             | 0.83            |
| **Income source**  |                  |                |               |                 |
| Agriculture        |                  | 88.3           | 95            | 91.6            |
| Government employed|                  | 3.33           | 1.6           | 2.5             |
| Pity trade         |                  | 8.33           | 3.3           | 5.8             |

N = Total sample % = Percentage
Table 2. Knowledge attitude and practice of household dairy farmers.

| Parameter                                      | Categories | Dale N = 120 | Hula N = 120 | Total N = 240 |
|------------------------------------------------|------------|--------------|--------------|---------------|
| Knowledge about aflatoxin.                     | Yes        | 2.5%         | 4.16%        | 3.3%          |
|                                                | No         | 97.5%        | 95.8%        | 96.6%         |
| If yes, source                                 | Public media | 0.8%         | 1.66%        | 1.25%         |
|                                                | Health extension | 1.66%       | 2.5%         | 2.1%          |
| Moldy feed treatment                           | Yes        | 43.3%        | 33.3%        | 38.3%         |
|                                                | No         | 56.6%        | 66.6%        | 61.6%         |
| Utilization of feeds and food leftover         | Throwaway  | 76.6%        | 75%          | 75.8%         |
|                                                | Feed to animal | 23.33%     | 23.3%        | 23.3%         |
|                                                | Human consumption | -     | 1.6%         | 0.8%          |
| Type of animal consuming                       | Chicken    | 18.3%        | 10%          | 14.2%         |
|                                                | Cow        | 5%           | 11.6%        | 8.3%          |
|                                                | Donkey     | -            | 1.6%         | 0.8%          |
| Exposure to training on handling and storage of animal feeds | Yes | 3.3% | 15% | 9.2% |
|                                                | No         | 96.6%        | 85%          | 90.8%         |
| Factors encouraging mold formation?            | Moisture content | 33.3%     | 48.3%        | 40.8%         |
|                                                | Temperature | 5%           | 6.6%         | 5.8%          |
|                                                | Storage length | 13.3%     | 10%          | 10.8%         |
|                                                | Humidity   | 26.6%        | 10%          | 18.3%         |
|                                                | All        | 21.6%        | 25%          | 23.3%         |
| Effect of feeding moldy feed to animals        | Yes have problem | 70%         | 73.3%        | 71.6%         |
|                                                | No problem | 8.3%         | 20%          | 14.2%         |
|                                                | I don't know | 21.6%     | 6.6%         | 14.2%         |
| Moldy food consumption on human health         | Yes have problem | 63.3%     | 73.3%        | 68.3%         |
|                                                | No problem | 25%          | 20%          | 22.5%         |
|                                                | I don't know | 11.6%     | 6.6%         | 9.2%          |

N = Total sample % = Percentage

Small proportion of the respondents (9.2%) attended training on animal feed harvesting and storage indicating the need for giving training and awareness creation on feed management and quality maintenance-related issues. Respondents identified moisture, humidity, temperature and storage length as major factors that encourage mold formation. Majority of the farmers (71.7%) reported that feeding moldy feeds and food leftover for animal has health effect like appetite reduction, emaciation and diarrhea. Small proportion of the respondents did not know if feeding moldy feed had any effect on animals. The respondents (68.3%) also believe that feeding mold and food left over for animal and using their product like milk and meat had health effect for human.

Concentration of aflatoxin M$_1$ in cow milk in the study area

Under the current study, out of 240 raw milk samples collected from the two districts 77.91% had detectable level of aflatoxin M$_1$ while the rest were below the detection limit of the ELISA detection method. The detected samples recorded aflatoxin concentration range of 0.00–0.038 μg/L in the milk with an average of 0.0068 μg/L (Table 3). Only 9% of the milk samples were above EU limit (0.025 μg/L) for infant consumption and 16.6% of milk samples were above FAO limit of 0.01 μg/L for infant milk consumption in some countries. The result of the current study is similar to the study by [24] who reported all milk samples from rural dairy system had

Table 3. AFM$_1$ in milk concentration during wet and dry season in the study area.

| Season  | N  | Positive(N) | Mean ± SD (μg/L) | Range (μg/L) | P     |
|---------|----|-------------|------------------|--------------|-------|
| Wet     | 120| 88          | 0.0049 ± 0.0041  | 0.00–0.021   | P = .001 |
| Dry     | 120| 99          | 0.0085 ± 0.0093  | 0.00–0.038   |       |
| Total   | 240| 187         | 0.0069 ± 0.0075  | 0.00–0.038   |       |

N = total sample SD = standard deviation. ug/L = Microgram per litter.
AFM₁ contamination below the EU limits ranging between 0 and 0.041 μg/L. Similarly, in Iran also report 0.01161 ± 0.00072 μg/L concentration of AFM₁ in raw milk with a range of 0.0063–0.0233 ppb. None of the samples had levels exceeding the maximum tolerable limit (0.05 μg/L) accepted by the European Union. In contrast, the result of the current study is lower than the report of in the rural area of Pakistan where out of 48 samples analyzed 52% samples were contaminated with range of 0.004 to 0.71 μg/L AFM₁ in which 27% of the samples were above EU limit. A survey conducted in Nigeria in 2014 reported the incidence of AFM₁ contamination in fresh cow milk from free grazing cows to be 0.009–0.456 μg/L where 64% of the positive samples exceeded the limit set by the European Union. Another study conducted in small scale dairy farms in Zimbabwe indicated the level of AFM₁ contamination in the range of 0.74–1.30 μg/L where only 30% of the milk samples were positive. A study by in rural area of South Africa also reported that 100% of the milk samples were contaminated having values ranged from 0.02 to 0.15 μg/L and means of 0.082 μg/L where 68.65% of samples were above EU limit (0.05 μg/L).

In addition to these, the result obtained in the current study is lower than the previous study conducted both in the rural and urban areas in Ethiopia, in urban area (Addis Ababa), the above authors reported a concentration ranged from 2.34 to 76.07 μg/L with an average content of 29.3 ppb. The same study in rural areas of Sululta, Debrebirhan and Bishoftu showed 0–7.57 ppb with an average aflatoxin content of 1.98 ppb using high performance liquid chromatography (HPLC). An AFM₁ concentration level of 0.028–4.98 μg/L and average 0.41 μg/L by ELISA detection method in the Greater Addis Ababa milk shade. Another study carried out in Bishoftu, Holeta and Hawassa (tabor sub city and monopol) sites also exhibited an average AFM₁ concentration of 0.088, 0.057 and 0.017, respectively, with overall average of 0.054 μg/L by ELISA. Furthermore, a study carried out in Gurage zone reported the AFM₁ concentration in milk with a range of 0.02–0.31 μg/L and an average content of 0.11 μg/L.

The variations of AFM₁ concentrations in milk and dairy products in different countries might be due to the differences in the geographical location, climatic factors, seasons and e-storage of animal feed and the application of good manufacturing practice in primary production. The other factor that affect variation in AFM₁ concentrations are animal health, milk yield and lactation stage which determine the carry over rate of AFB₁ to AFM₁. The reason of low AFM₁ concentration in the current study might be due to the farmer’s usage of fresh roughage feed throughout the year due to availability of enset (enset ventricosum) in the study area, and shorter storage time of animal feed besides the lower milk yield of the local cows (<30 L), which results in lower carryover rate of AFB₁ to AFM₁.

**AFM₁ concentration in milk during wet and dry season**

Aflatoxin concentration in raw milk in the dry season was significantly higher (P < .05) than wet season. The minimum and maximum concentration during wet season were 0.00 and 0.021 μg/L with mean 0.0049 ± 0.0041 μg/L which is significantly lower than dry season minimum and maximum concentration 0.00 and 0.038 ppb, respectively, with mean of 0.0085 ± 0.0093 μg/L. (Table 3). This might be due to available feed and feeding. During the wet season, fresh grazing feeds are highly available while stored crop residues, hay and maize stovers, agro industrial by products especially wheat bran are most commonly used in the dry season of the study, which are more susceptible for mold formation due to moisture contents and storage durations. The finding of the current study is similar with the results reported in local markets of Karachi, Pakistan who reported that aflatoxin M₁ concentration in milk during dry season (May–August) were higher than wet season. However, in contrast with the present study, several previous reports in different countries like Iran, China and Serbia reported significantly higher AFM₁ milk concentration during the wet season (November to March) than the dry season (June to August).
Aflatoxin M₃ in milk in different location in the study area

As described in Table 4, average aflatoxin M₃ concentration in raw milk of Hulla site (0.0088 ± 0.0088 µg/L) is significantly (P < .05) higher than Dale site (0.0051 ± 0.0056 µg/L). This could be due to the relatively higher AFB₁ concentration in feed in the Hula site which results due to feed and feeding differences. Higher number of respondent from Hula site use dry and stored feed (Table 2). They store for more than a month when compared with Dale site which is similar with a study reported in[17] in addition the higher concentration in the Hula site probably related with the common use of maize stover and supplementary concentrate feed in the diet of animal these types of feed are prone to fungal infection during storage and moisture content of the location facilitate the mold growth in feed. Those factors increase possibility of AFB₁ production which, subsequently higher in aflatoxin M₃.

Interaction effects of location and season on aflatoxin content

Interaction effect of season and location on AFM₃ in milk (Table 5) indicated that both single factors alone and interactively significantly (P ≤ .001) affected AFM₃ concentration in the study area. This might be due to variation of feed types susceptibility like maize and concentrate feeds are highly susceptible,[15] animal conditions (health, milk yield, lactation stage and breed types) which cause variation of carry over rate of AFB₁ to AFM₃,[33] environmental temperature variation which related with mold growth possibility in feed[38] and other factors cause concentration of aflatoxin B₁ difference, subsequently difference in AFM₃.

Conclusion

This study assessed the awareness level of dairy farmers about aflatoxin and evaluated the occurrence and level of aflatoxin M₁ contamination in raw cow milk. Majority of respondents have no awareness against aflatoxin. The laboratory analysis showed 77.91% of collected raw milk samples had detectable
level of aflatoxin M1. However, none of the samples exceed the EU and USA standard which makes them safe for human consumption. The milk samples collected in dry season of the study area exhibited significantly higher aflatoxin M1 concentration than the wet season. This indicated that considering the seasonal factors and awareness creation about aflatoxin played a key role for the control of aflatoxin in raw milk.

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No potential conflict of interest was reported by the author(s).

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**Data availability statement**

Data will be made available on request.

**Declaration**

The authors declare no conflict of interest.

**Consent to participate**

Verbal informed consent was obtained prior to each farmer’s interview.

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