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Aflatoxin Occurrence in Dairy Feeds: A Case of Bulawayo, Zimbabwe

Nancy Nleya, Lubanza Ngoma and Mulunda Mwanza

Abstract

Aflatoxin contamination in feeds used by Bulawayo peri-urban farmers for dairy cows was assessed. Semi-intensive farming was the most common farming type practised by the farmers where the animal feeds were supplemented with mixed rations, concentrated feed, grass and brewers' spent grains. Mixed ration was the most commonly used feed supplement. Feed analysis by high-performance liquid chromatography (HPLC) showed the presence of all four naturally occurring aflatoxins: aflatoxins B₁, B₂, G₁ and G₂. Total aflatoxin concentration in the feeds ranged from 0 to 250.9 µg/kg. Mixed ration had the highest average total aflatoxin concentration of 29.0 µg/kg, which is above the European Union (EU) standard adopted by Zimbabwe. AFB₁, the most potent aflatoxin was the predominant aflatoxin across all feeds with an average concentration of 9.0 µg/kg and highest concentration of 149.6 µg/kg in a mixed ration sample which is also above the EU 5.0 µg/kg for lactating cows. Farm personnel responses to the questionnaire showed that most of them were not aware of aflatoxins. These findings call for stringent measures to be put in place with regard to aflatoxin testing in feeds for the dairy sector as well as educating the farmers on the importance of aflatoxin monitoring feed ingredients and livestock feeds.

Keywords: aflatoxins, feeds, dairy, cows, chromatography, farming systems, monitoring

1. Introduction

Animal feed ingredients are at risk of mould contamination with subsequent mycotoxin production during preharvest, harvest and postharvest times [1–3]. The sources of the individual components used in the formulation of dairy feeds are quite diverse ranging from cereals, cereal products, oil seeds as well as hay and forages [3, 4]. Also the high cost of feed has led to the addition of stale bread, kitchen and bakery wastes to the feed. Furthermore scarcity of protein sources for animal feeds has led to the use of alternative protein sources such as brewers' spent grains (BSG) [5]. These waste products are usually tainted with fungus and may be a contributing factor in mycotoxin production in cattle feed. Aflatoxins are the most toxic mycotoxins produced by members of

the genus *Aspergillus* [6], and their presence in animal feedstuffs has become a potential health hazard to both animals and humans [7]. Toxic effects of aflatoxins in ruminants include liver damage, diminished growth efficiency, diminished milk production and quality and impaired resistance to infectious diseases [7–9].

In dairy farming, depending on the farming system adopted, the diet consists of the concentrates, alternative protein sources as well as forage; hence the animals are exposed to more than one type of mycotoxins [4]. Although there are more than 20 aflatoxins known, only four of these occur naturally, namely, aflatoxins (AF) B₁, B₂, G₁ and G₂, based on their fluorescence under UV light (blue or green) [10–12]. The most abundant aflatoxin in cow feeds and rations is aflatoxin B₁ and is also the most potent of them all [13, 14].

Animals differ in their sensitivity to mycotoxin toxicity [15] with ruminants being more resistant than the monogastrics [16] mainly because they have microorganisms in their rumen which play significant roles in the deactivation and degradation of the aflatoxins as well as alteration of the binding of the aflatoxins to some essential nutrients [17, 18]. However, aflatoxins are poorly degraded by ruminants as most of the rumen microbiota are inhibited by AFB₁ concentration of 10 µg/ml [16]. The aflatoxins will get to the bioconversion sites of nutrients and xenobiotics like the intestinal epithelium, liver and kidneys unaltered [16]. In the liver, AFB₁ is bio-transformed to AFM₁ which enters the circulatory system or is conjugated to glucuronic acid. The conjugated AFM₁ is excreted through the biliary system, and the one in circulation may be excreted through urine and milk. It has been shown that AFM₁ retains some carcinogenic activity resulting in its reclassification by IARC as a group 1 carcinogen [19–21]. Consumption of AFB₁-contaminated feed by lactating cows results in its metabolism into AFM₁ subsequently secreted into milk thereby making milk a source of aflatoxin contamination in humans. In this study the extent of aflatoxin contamination of feeds used in different feeding systems adopted by dairy farmers was assessed.

2. Methodology

2.1 Data collection

Convenience sampling coupled with snowball sampling methods was used to identify farmers willing to participate in the research. Questionnaires were used to get information from the farmers. The information required from the farmers included the following: plot size in acres, number of cattle owned by the farmer, number of cows that were being milked, age, breed, lactation stage, milking method, volume of milk produced on the farm per day, volume of milk produced by each cow per day, number of milking per day, amount of feed given to each cow per day and also if the farmer had any knowledge on aflatoxins. A total of 14 farmers participated in this study with farm size of 8.5 hectares and above. Most of them were milking cows ranging between 20 and 250, and a few had less than 10 cows. The cows that were being milked were 25 months old and above, and the common breeds were the Jersey, Holstein and crossbreed (Holstein/Jersey) across all milking stages. Majority of the farmers were milking by hand getting a volume of 100 to over 200 litres per farm per day with each cow giving an average of 6–10 litres.

2.2 Sample collection

A total of 96 feed samples which consisted of dairy feed concentrates (CN), mixed ration (MR), brewers spent grain (BSG) and grass (GR) were collected from 13 farms during the dry season (August–October 2016) and the rainy season (January–March 2017). Samples were collected in sterile polythene ziplock bags which were sealed and transported in cooler boxes to the laboratory where they were ground to a fine powder using IKA® M20 universal batch mill (Germany) and stored in the freezer at -20°C until time for analysis [22].

2.3 Sample preparation for HPLC analysis

Aflatoxins from feeds were extracted using the immunoaffinity extraction method [23] using Easi-Extract® aflatoxin immunoaffinity columns (R-Biopharm Rhone Limited, Glasgow G20 OXA, Scotland). Extraction was carried out according to the manufacturer's protocol with some modifications as follows: a portion of 50 g of the sample was mixed with 5 g of sodium chloride (NaCl) in a laboratory blender followed by 100 ml of methanol: water (80:20 v/v) and blended for 5 minutes. The mixture was filtered through a fluted filter paper (Whatman No.1) into a clean vessel. A volume of 2 ml of the filtrate was then diluted with 14 ml phosphate buffer saline (PBS) solution and passed through an immunoaffinity column. The column was washed with 20 ml of PBS and the aflatoxins finally eluted with 1 ml methanol (LiChrosolv®, Merck, Germany) into a glass cuvette and diluted with 1 ml of distilled water and then stored at -20°C prior to analysis. Aflatoxin B₁, B₂, G₁ and G₂ standards (Trilogy Analytical Laboratory, Washington, USA) were diluted using acetonitrile (LiChrosolv®, Merck, Germany) to give the following concentrations: 5×10^{-6} , 5×10^{-5} , 5×10^{-4} , 5×10^{-3} and 5×10^{-2} mg/ml. Aflatoxin detection and quantification were done using HPLC (Shimadzu FCV-20H2) with operation conditions as given in the KOBRA® cell instruction manual as follows: derivatisation using KOBRA® cell at 100 μA setting, with an analytical column Inertsil ODS-3 V 5 μm , 4.6×150 mm equipped with a C18 4×3 mm² ID security guard cartridge (Phenomenex, Torrance, CA, USA). Mobile phase was modified from the recommended water: methanol (60:40) to a working condition of 55:45 with 119 mg/litre of potassium bromide (KBr) and 1 ml/litre of 65% nitric acid added at a flow rate of 1.0 ml/minute, and fluorescence detector is set at 362 nm for excitation and emission 425 nm (AFB₁ and B₂) and 455 nm (AFG₁ and G₂). Injector was an auto sampler which injected 100 μl of sample, and elution of the aflatoxins was in the order (AF) G₂, G₁, B₂ and B₁.

Calibration curves for each aflatoxin, AF (B₁), B₂, G₁ and G₂, were constructed using standard solutions which were diluted with acetonitrile to give the following concentrations: 0.005, 0.05, 0.5, 5 and 50 $\mu\text{g/kg}$. The limit of detection for all the standards was 0.005 $\mu\text{g/kg}$. The linearity of the standard curves was determined using correlation regression (r^2). A curve with good linearity will have an r^2 value close to 1. Aflatoxin concentration of the samples was calculated by measuring the area of the peak and then interpolating from the standard curve.

2.4 Statistical analysis

Descriptive statistics was used to show the distribution of aflatoxins in the different feeds and one-way ANOVA used for significance testing using IBM SPSS Statistics 25.

3. Results

3.1 Farmer survey

Most of the farmers who took part in the study were practising semi-intensive farming followed by extensive and lastly intensive farming as summarised by **Figure 1**.

The cows were mainly fed with concentrates, mixed ration, brewers’ spent grain and grass ranging from 6 to 10 kg per animal per day. Only 36% of the farmers had some knowledge on aflatoxins. The most utilised feed was mixed ration as shown by **Figure 2**.

3.2 Analysis of aflatoxins

HPLC analysis of aflatoxins showed the presence of all the major aflatoxins AF (B₁), B₂, G₁ and G₂ in the bulk of the samples indicated by the peaks in the

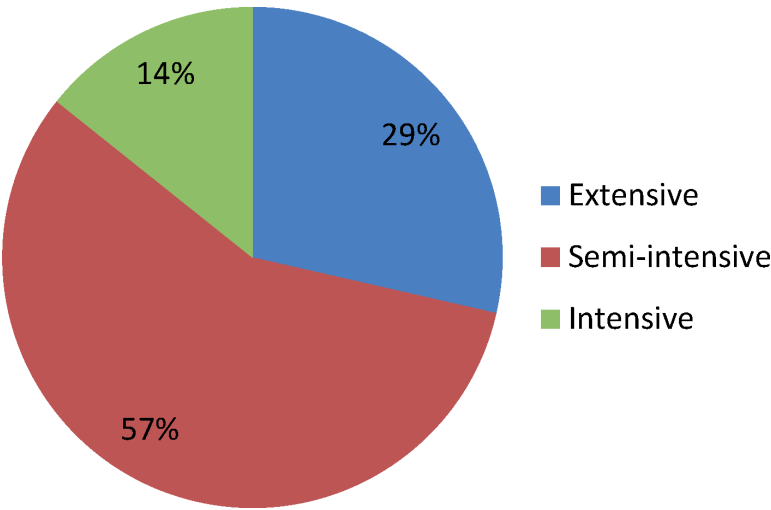


Figure 1.
Farming systems adopted by dairy farmers in Bulawayo peri-urban showing that most the farmers practise semi-intensive farming.

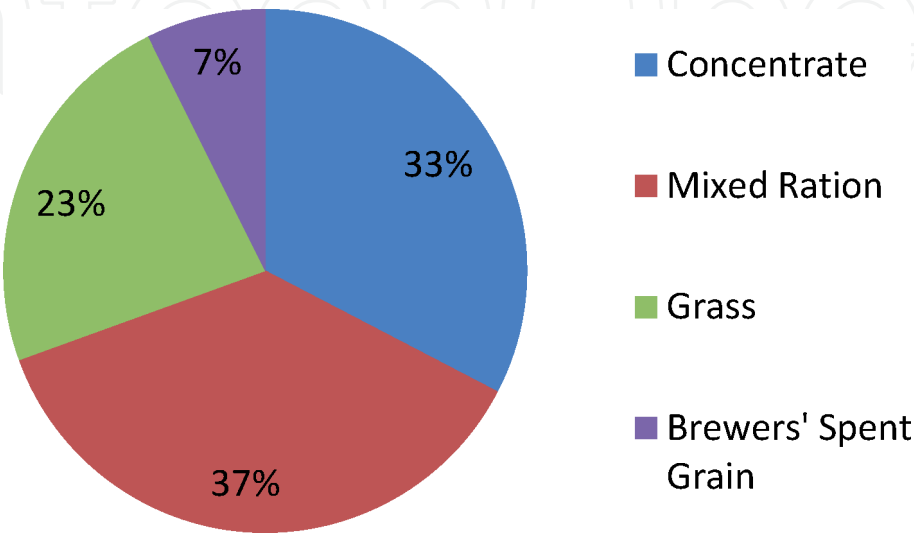


Figure 2.
Percentage utilisation of feed types by dairy farmers in peri-urban Bulawayo showing that the most common feed used by the farmers is the mixed ration.

chromatograms as shown in **Figure 3**. The calibration curves gave good linearity for the different aflatoxins with r^2 values of 1. Total aflatoxin concentration in the feeds ranged from 0 to 250.9 $\mu\text{g}/\text{kg}$.

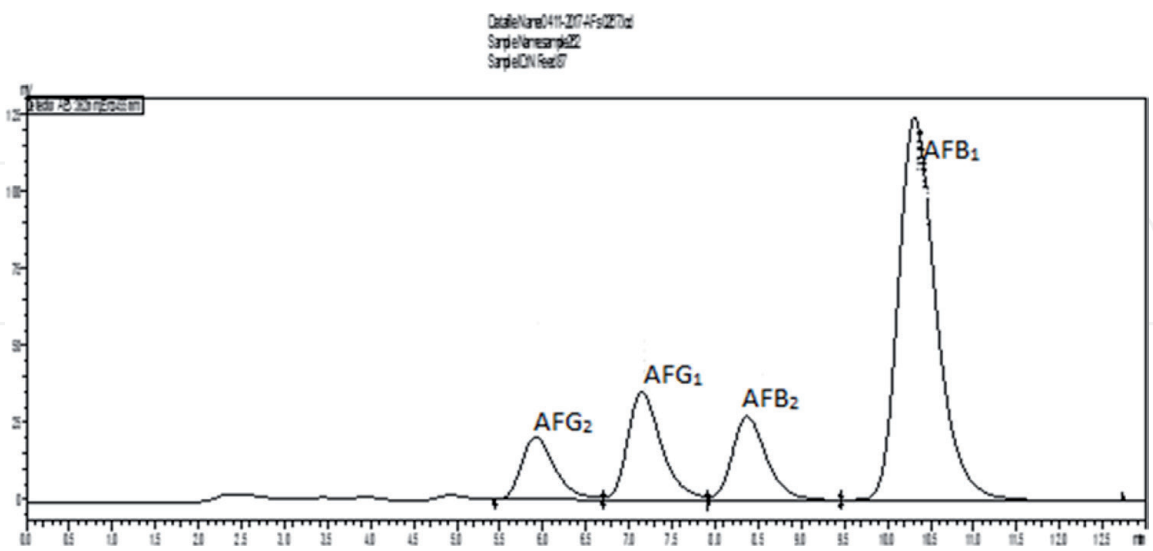


Figure 3.
Representative chromatogram showing four peaks indicating the presence of all major aflatoxins.

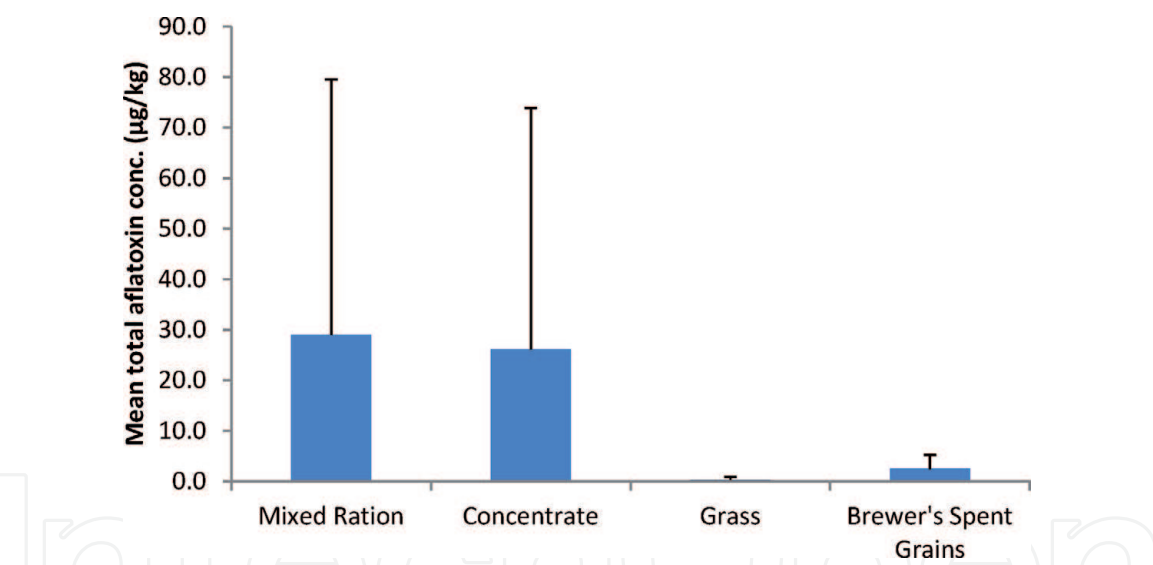


Figure 4.
Average total aflatoxin concentrations in the feeds. A p value of 0.043 shows that there was significant difference in the aflatoxin concentrations in the different feeds with mixed ration had the highest contamination.

ANOVA					
Total AF conc (ug/kg)					
	Sum of squares	df	Mean square	F	p value
Between groups	14860.674	3	4953.558	2.832	0.043
Within groups	159185.082	91	1749.287		
Total	174045.756	94			

A p value <0.05 indicates that there is a significant difference in the levels of aflatoxin in the different types of feeds used for feeding the dairy cows.

Table 1.
One-way ANOVA for all feed types.

3.3 Aflatoxin distribution in feeds

Mixed ration had the highest total AF concentrations with an average concentration of 29.8 µg/kg, and grass had the lowest concentrations as shown in **Figure 4**. The one-way analysis of variance (ANOVA) (**Table 1**) gave a *p* value of 0.043, meaning that at 95% confidence level (*p* < 0.05) there is enough evidence to conclude that there is a significant difference in the total mean concentration of aflatoxins across the feeds. However, looking at MR and CN (**Table 2**), *p* = 0.766; therefore there was no significant difference in the mean total aflatoxin concentrations.

The distribution of aflatoxins in the feeds showed that AFB₁ was the most common aflatoxin across all feeds as shown by **Figure 5**. However, there was variation with individual feeds as shown in **Figure 6a–d**.

Looking at the distribution of total aflatoxins across the different farming systems, **Figure 7** shows that the semi-intensive system had the highest aflatoxins with an average of 21.6 µg/kg. One-way ANOVA (**Table 3**), however, indicated that there is no significant difference in the mean total aflatoxin concentration in the feeds from semi-intensive and intensive farming systems as *p* = 0.937 which is greater than *p* value of 0.05 at 95% confidence level.

ANOVA					
Total AF conc (µg/kg)					
	Sum of squares	df	Mean square	F	<i>p</i> value
Between groups	218.928	1	218.928	0.089	0.766
Within groups	159133.265	65	2448.204		
Total	159352.193	66			

A p value >0.05 indicates that there is no significant difference in the levels of aflatoxin.

Table 2.
One-way ANOVA between the mixed ration and feed concentrate.

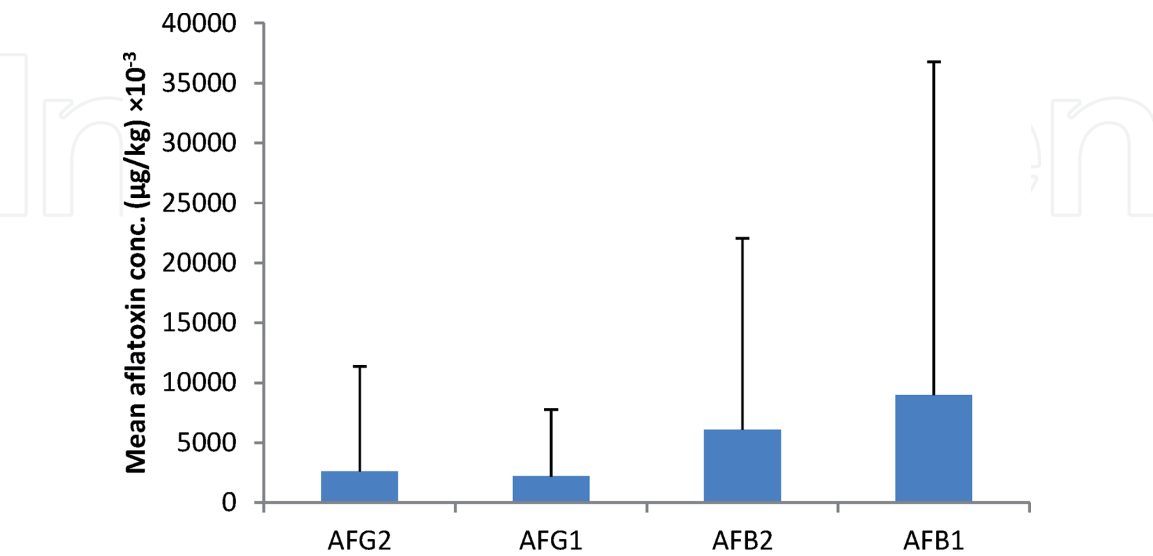


Figure 5.
Distribution of aflatoxins across all feed types. One-way ANOVA analysis gave a *p* value of 0.017, indicating a significant difference between the concentrations of the individual aflatoxins with AFB₁ being the most dominant aflatoxin.

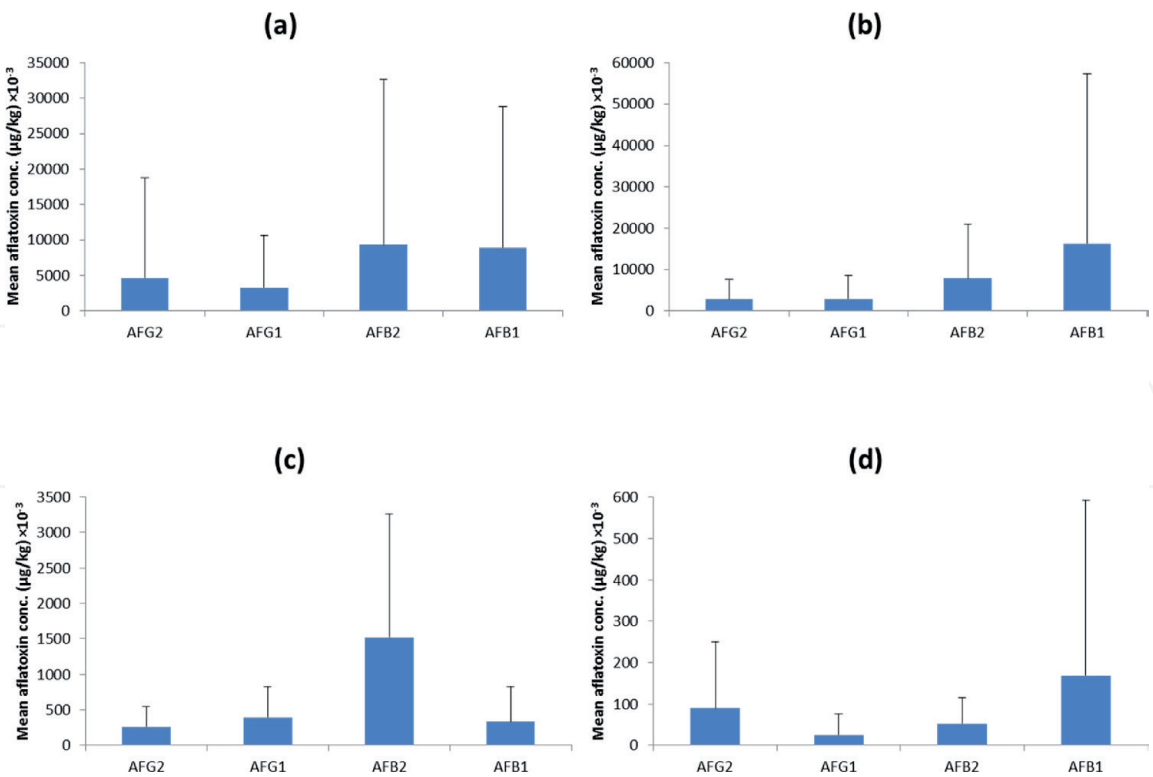


Figure 6. Distribution of aflatoxins in the feeds, (a) feed concentrates, (b) mixed ration, (c) brewers' spent grains and (d) grass. One-way ANOVA gave a p value of 0.017, indicating a significant difference in the concentration of individual toxins across all feeds. AFB₁ was the dominant aflatoxin in mixed ration and grass, whereas for concentrates and brewers' spent grains, AFB₂ was the predominating aflatoxin.

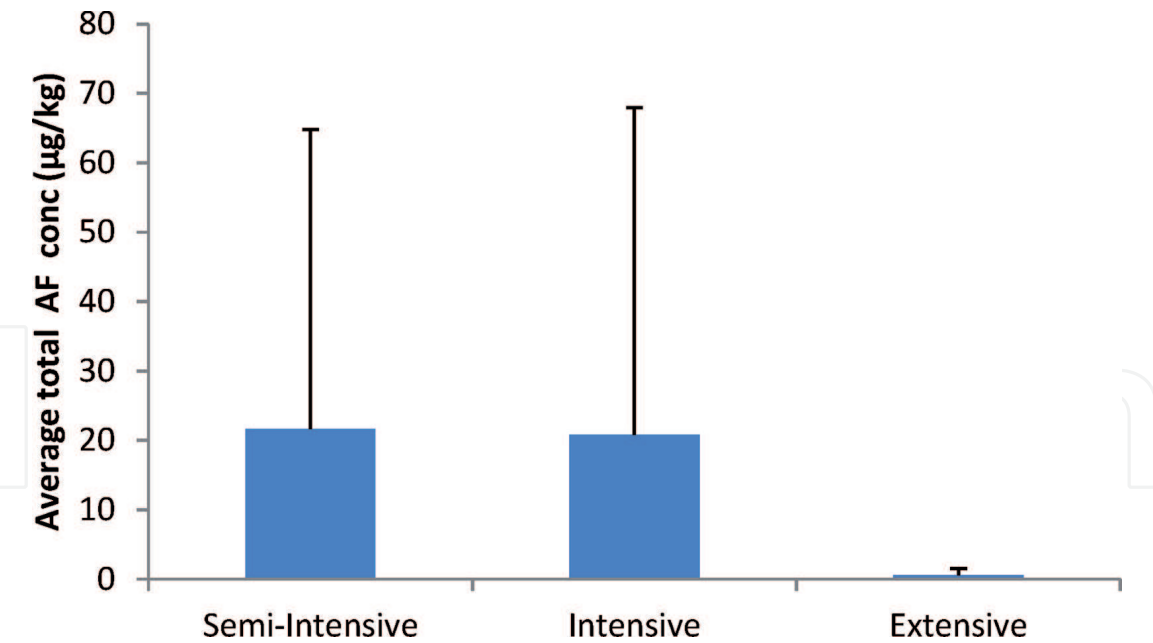


Figure 7. Distribution of aflatoxins across the three farming systems. Descriptive statistics shows that extensive farming has the lowest aflatoxin concentration and semi-intensive farming has more aflatoxin concentrations in their feeds. However, one-way ANOVA gave p = 0.470, indicating no significant difference among the different farming systems.

Distribution of AFB₁ in the feeds from the different dry and rainy seasons is shown in **Figure 8**, and ANOVA analysis showed that there is a significant difference in AFB₁ concentrations in the different seasons (**Table 4**).

ANOVA					
Total AF conc (µg/kg)					
	Sum of squares	df	Mean square	F	p value
Between groups	12.581	1	12.581	0.006	0.937
Within groups	171580.359	87	1972.188		
Total	171592.940	88			

A p value >0.05 indicates that there is no significant difference in the levels of aflatoxin concentration between.

Table 3.
One-way ANOVA results comparing the semi-intensive and intensive farming systems.

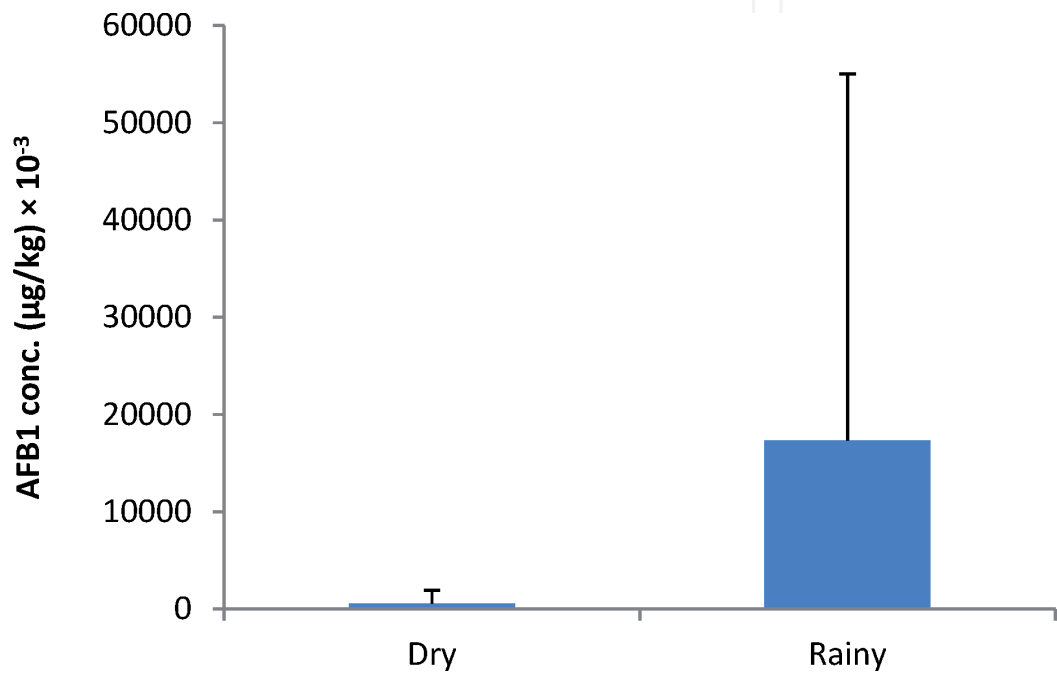


Figure 8.
Seasonal variation in the distribution of AFB₁. There was a significant difference in AFB₁ concentrations (p = 0.003) with samples from the rainy season having more of AFB₁ than dry season samples.

ANOVA					
AFB ₁ conc (µg/kg) × 10 ⁻³					
	Sum of squares	df	Mean square	F	p value
Between groups	6747185610.100	1	6747185610.100	9.500	0.003
Within groups	66758340020.045	94	710195106.596		
Total	73505525630.144	95			

A p value <0.05 indicates that there is a significant difference in the levels of AFB₁ concentration in feeds in the rainy season.

Table 4.
One-way ANOVA results for dry season and rainy season.

4. Discussion

Feed quality is of great importance in animal husbandry as it affects both animal health and productivity [24]. Consumption of aflatoxin-contaminated feeds by dairy cows may result in the aflatoxins occurring in milk posing health risks to

humans [18]. Research has shown that some feedstuffs used in formulating animal feeds can become infected by aflatoxin-producing fungi [25]. Researchers worldwide have been analysing dairy feed for aflatoxin contamination and have reported various findings with most feeds exceeding the regulatory limits [26–29].

This study also showed that 96% of feeds used in feeding dairy cows in peri-urban Bulawayo that were analysed were contaminated with at least one of the naturally occurring aflatoxins. The results also indicate that 21% of the samples analysed had total aflatoxin levels above the regulatory limit set by international governing bodies of 20 µg/kg for animal feeds. This concurs with the findings by Reddy and Salleh [30] who reported that 22.5% of their samples had aflatoxin concentrations above this regulatory limit. Zimbabwe reviewed the AFB₁ regulatory limit to 20 µg/kg in 1990 [31] for food intended for human consumption. However, there are no regulatory limits in terms of animal feeds [32].

The feeds that are used in feeding dairy cows by farmers in peri-urban Bulawayo included feed concentrates, mixed ration, grass and brewer's spent grains. This is in accordance with the requirements of the diets of dairy cows which should consist of a component that provides protein and energy and a component of roughage [33]. In this study, the protein and energy were supplied by the concentrates, mixed ration and the brewer's spent grains, whereas the roughage was provided in the form of hay stored at the farm or fresh grass in the grazing land.

Mixed rations are considered a whole meal for the cow as they contain basically all the nutrients that are found in forages and concentrates. Formulation of a mixed ration involves combining forages, by-products of other processes such as whole cottonseed or cottonseed cake, grains, protein source, minerals and vitamins [34]. Findings of this study showed that mixed ration had the highest total aflatoxin concentrations with an average of 29.0 µg/kg. ANOVA also showed that at 95% confidence level, there was a significant difference in the mean total aflatoxins in the feeds with the mixed rations having the highest total aflatoxin mean. Findings from this study concur with Mozafari et al. [35] who detected the highest aflatoxin concentrations in mixed ration among the other feeds they analysed. The diversity of the components used could have been potential sources of aflatoxigenic fungi which result in contamination of this feed type with aflatoxins. Other researchers [25] also reported high aflatoxin concentration in noug cake, a product of oil processing industry used in feeding dairy cows. Cottonseed was the most utilised feed ingredient for mixed rations by the farmers who participated in this study. However, Chohan et al. [36] reported feed concentrate having the highest aflatoxin concentration followed by mixed ration in their study on aflatoxin contamination of different feeds and feed ingredients used to feed dairy cows in Pakistan.

From this study it was shown that grass samples had the least aflatoxin concentrations with an average total aflatoxin concentration of 2.5 µg/kg and 169×10^{-3} µg/kg of AFB₁. These results are similar to the finding by Gizachew et al. [25] who also had grass as the least contaminated feed. However, they got a minimum AFB₁ concentration 7 µg/kg for their samples, higher than what was established in this study. Sassahara et al. [37] analysed feedstuffs supplied to dairy cows in North of Paraná state, Brazil, and did not detect any aflatoxins in the silage samples. Work done by Driehuis et al. [33] in the Netherlands also showed the absence of aflatoxins in silage samples used to feed dairy cows. These findings suggest that grass in the form of silage or pasture is not really prone to fungal infections which may result in aflatoxin production. In this study most of the aflatoxigenic strains were isolated from the grass, but it was the feed with the least aflatoxin concentration. Gonzalez Pereyra et al. [38] highlighted that the presence of aflatoxigenic fungi on a substrate does not mean that the toxin is present in that particular food/feed matrix, but there is a risk of toxin production if the environmental conditions become favourable

for aflatoxin production. Nonetheless, detection of aflatoxins in a sample means the substrate has been contaminated by toxigenic species which could either be present or absent at the time of sampling. This was the case with the feed concentrates which had aflatoxin concentrations higher than the grass samples, but fewer toxigenic strains were isolated.

The most dominant aflatoxin across all feeds was AFB₁ with an average concentration of 9.0 µg/kg and was detected in all the samples that tested positive for aflatoxin contamination. This is above the EU 5 µg/kg set for lactating cows. Udom et al. [39] and Gizachew et al. [25] also reported their samples having AFB₁ concentrations exceeding the EU regulatory limit. The high levels of AFB₁ in most samples could be attributed to the fact that it was the most common and prevalent aflatoxin in most food matrices [40, 41]. Moreover, some authors have indicated that most toxigenic *Aspergillus* strains produce AFB₁ and therefore it occurs more frequently than the other aflatoxins [10, 42, 43]. AFB₁ was predominant in the rainy season (**Figure 8**). These results are in agreement with the findings by Chohan et al. [36] which also showed high concentrations of AFB₁ during the rainy season. For aflatoxin production, high temperatures and high humidity are required, and these conditions prevail during the rainy season.

However, for brewers' spent grains (BSG), AFB₂ was the predominant aflatoxin. The BSG are a product of beer brewing industry [44] and has been found to be of valuable use in the feedstock industry mainly because it is affordable and available throughout the year [45]. BSG used in this study were from the production of opaque beer. The presence of aflatoxins in beer production has been associated with contaminated malt. Malt production involves increasing the moisture content of the grains to allow partial germination of the grain. Aflatoxigenic fungi are known to contaminate cereal grains which are also used in the beer production process [46]. If the malt is not properly dried or stored, fungal growth may be promoted resulting in the production of aflatoxins. Research on the fate of mycotoxins during the beer fermentation process showed that recovery of AFB₂ in BSG is higher than other aflatoxins [47]. Some researchers [48] showed that AFB₂ is able to adsorb onto yeast cells during fermentation. The yeast cells and the grain particles that are removed through filtration are collectively known as brewers' spent grains. This could be the possible reason why AFB₂ levels were higher in BSG samples. Nevertheless, Gonzalez Pereyra et al. [38] were not able to detect any AFB₂ in barley malt and brewers' spent grains from Argentina breweries. AFB₁ has been reported as the most common aflatoxin occurring naturally in feedstuffs, but for this study it was not the case for BSG as the concentration of AFB₂ was higher than that of AFB₁.

This study also showed that aflatoxin contamination of brewers' spent grains, a known source of nitrogen and roughage, and grass were within the regulatory limits making them safer when compared to the concentrates and mixed ration. However, nutritional composition of the grass will not meet the dietary demands of the cows.

5. Conclusion

Detection of aflatoxins in the feed samples used for this study is a cause of concern as this may be indicating the possibility of transfer into the milk by the dairy cows. Although most samples were within the acceptable limit for total aflatoxin, it was noted that concentrations of AFB₁, the most potent of them, were above the regulatory limit. Moreover, research has shown that AFB₁ can be carried over into milk as its hydroxylated metabolite AFM₁ making milk a route through which humans are exposed to aflatoxins. High prevalence of AFB₁ during the rainy season could be an indication of poor storage of the feeds which may result in increased

moisture content resulting in proliferation of aflatoxin-producing *Aspergillus*. Therefore, there is a need to educate the farmers and their personnel on the importance of proper feed storage facilities in order to control contamination of the feeds.

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Conflicts of interest

The authors declare no conflict of interest.

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