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Seasonal variation of aflatoxin B_1 content in dairy feed

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ABSTRACT. Aflatoxin B_1 (AFB_1) is a fungal metabolite and highly carcinogenic compound of category 1 according to the International Agency for Research on Cancer. In the liver AFB_1 from contaminated feed is bioconverted into aflatoxin M_1 and can be easily diffused to the animal milk. Provision of healthy milk for humans, particularly infants and adults, therefore, entails monitoring of AFB_1 level in the feed for dairy animals. In the present study, AFB_1 level was monitored in three different types of animal feed comprising commercially available animal feed, fresh fodder and leftover bread fed to dairy animals between October 2014 and September 2015. AFB_1 was found in all collected feed samples at the amounts: 30.5%, 2.8% and 88.9% in commercial feed, fresh fodder and leftover bread samples, respectively. All these levels were over the EU permissible limits (5 μg · kg^{-1}). Mean maximum levels of AFB_1 were observed in all samples collected in the winter season, whereas the mean minimum levels – in the summer months. The results of the present study indicated that the leftover bread samples and commercial feed contain high levels of AFB_1, and so strict measures should be adopted to prevent dairy animal feed and at the same time the animal milk from aflatoxin contamination.

Introduction

Aflatoxins are highly toxic secondary metabolites produced by different species of fungi such as *Aspergillus fumigatus, A. flavus, A. paraciticus, A. niger, A. tamari* and *A. nominus* (Ismail et al., 2017). They were discovered in 1960's in connection with the death of thousands of turkeys fed contaminated groundnut feed (Eaton and Groopman, 1994). Among 18 different types of aflatoxins, AFB_1 is said to be the most toxic. AFB_1 is reported to be teratogenic and mutagenic (Kang'ethe and Lang'a, 2009) and is categorized as group 1 carcinogenic compound by the International Agency for Research on Cancer (IARC, 2002). On the basis of toxicity aflatoxins are categorized as $B_1 > G_1 > B_2 > G_2$. AFB_1 has been detected in a number of food commodities including cereals such as maize (Karami-Osboo et al., 2012), rice (Reddy et al., 2009) and barley (Mateo et al., 2011). Moreover, AFB_1 has also been found in poultry (Bibin Becha and Devi, 2013) and other animal feeds (Britzi et al., 2013; Bagheri et al., 2014). Elevated level of AFB_1 in feed and food may increase the disease incidence in animals as well as

in humans. It may cause haemorrhage, DNA alteration, changes in metabolism and decreased resistance to infectious diseases. It also decreases milk production, deteriorates quality of milk and depresses growth rate of animals (Whitlow and Heagler, 2004; Akande et al., 2006). AFB_1 present in dairy animal feeds is biodegraded in the liver and is transferred into milk in the form of aflatoxin M_1 (AFM_1). Conversion factor of AFB_1 into AFM_1 has been estimated as 0.3 to 6% in animal model (Var and Kabak, 2009). Level of AFB_1 in the feed for dairy animals, therefore, is directly correlated with the presence of AFM_1 in the milk. As milk is consumed in large amounts by infants and elderly people – groups having low immunity level – the health agencies around the world monitor the presence of AFM_1 in milk and other dairy products.

Dairy industry suffers from reduced milk production, abortions and deaths of milking animals which are connected with the presence of mycotoxins in the animal feed, particularly AFB_1. Furthermore, stringent regulations governing the aflatoxin content in milk products imposed on milk producers led to the stoppage of milk and milk products export from the most of developing countries to other nations (Kaleibar and Helan, 2013; Chohan et al., 2016). Although the Pakistan dairy industry endures huge losses due to aflatoxin contaminated feed (Iqbal and Asi, 2013), it is still among leading producers of milk with ~38 million tonnes produced in the year 2012 (FAO, 2015). Environmental conditions, feed processing techniques, lack in awareness and ineffective monitoring agencies are the most probable reasons of high aflatoxin levels in animal feed. To produce safe milk in Pakistan the supply of safe feed to the milking animals is required. Despite the fact that high levels of aflatoxins in different types of dairy feed samples from Pakistan have recently been reported by Chohan et al. (2016) and Ullah et al. (2016), the systematic analysis of AFB_1 level at different seasons of a year is missing. So, the aim of the present study was to monitor the level of AFB_1 in the feeds from Southern Punjab (Pakistan) and to depict a clear picture of seasonal variations of these toxic substances.

Material and methods

Sample collection

Samples of feed for dairy animals were collected from five different districts of Southern Punjab (Pakistan) including Sahiwal, Multan, Dera Ghazi Khan, Rahim Yar Khan and Bahawalnagar. In total 216 samples, i.e. 72 for each feed type, were collected during four different seasons of the year (October 2014 – September 2015). Among feed samples were: commercial feed (%: maize grain 18, cotton seed cake 10, rapeseed cake 12, wheat bran 10, rice polishing 20, rice bran 6, sunflower cake 10, molasses 10, mineral mixture 2, $CaCO_3$ 1, urea 1), fresh fodder (seasonally available fresh grasses) and leftover bread (leftover bread from homes and/or hotels collected by street hawkers and sold to dairy farmers). All the feed samples were transported to the Food Analysis Lab of Institute of Food Science and Nutrition, Bahauddin Zakariya University, Multan (Pakistan) within 12 h. The samples were stored at 4 °C and analysed for AFB_1 within seven days after sample collection.

Chemicals and reagents

All the chemicals used in the experiment were of analytical grade. Good laboratory practices were adopted during the whole experiment.

Sample preparation

Sample preparation was done according to the Enzyme Linked Immuno Sorbant Assay (ELISA) kit for AFB_1 (Cat. No. 981BAFL01-96, Helica Biosystems, Santa Ana, California 92704, USA). Briefly, the samples were mixed and ground in automatic grinder (Mortar Grinder RM 200; Retsch, Dusseldorp, Germany). Ten gram sample was added to 50 ml of 80% acetonitrile and shacked vigorously for 3 min. The mixture was filtered through Whatman filter paper (No. 1, Whatman, Little Chalfont, UK) and diluted with 80% acetonitrile (1:2). The prepared solution was kept in refrigerator until analysis.

ELISA assay protocol

Enzyme Linked Immuno Sorbant Assay (ELISA) kit for AFB_1 (Cat. No. 981BAFL01-96) was purchased from Helica Biosystems (Santa Ana, California 92704, USA) and used according to the manufacturer's guidelines. The samples (100 µl of prepared solution) were run in duplicate. The absorbance of each sample, standards and blank were measured at 450 nm with the use of ELISA reader (ELx800, Bio-Tek, Winooski, VT, USA). The mean absorbance of standards and samples was divided by the absorbance of blank and multiplied by 100. AFB_1 concentration in feed samples was calculated on the basis of standard calibration curve.

Data evaluation

The mean values and standard deviations were calculated using Microsoft Office Excel (ver. 2013,

Microsoft, Redmond, WA, USA). For the statistical analysis of data Statistix 8.1 (Analytical Software, Tallahassee, FL, USA) software was used. Factorial analysis of variance (ANOVA) was performed to evaluate the differences among various feed types followed by post-hoc LSD test. The differences were considered statistically significant at $P < 0.05$.

Results and discussion

The highest levels of AFB_1 were found in all samples of feeds collected in the winter season, whereas in the samples collected in the summer season these levels were significantly lower (Table 1). The level of AFB_1 in the animal feeds can be presented as: leftover bread > commercial feed > fresh fodder. The maximum level of AFB_1 (8.9 ± 0.72 μg · kg^{-1}) was detected in the leftover bread samples during the winter season, while the minimum level of AFB_1 (2.1 ± 0.64 μg · kg^{-1}) was found in the fresh fodder during the summer season.

Table 1. Level of aflatoxins B_1 in dairy animal feed samples from October 2014 to October 2015

Season, month	n	Aflatoxin B_1 level		
		range, μg · kg^{-1}	mean, μg · kg^{-1}	above EU limit, %
Commercial feed				
autumn (Sep–Oct)	18	1.64–5.72	4.3 ± 0.21^{de}	22
winter (Nov–Jan)	20	2.30–9.76	7.3 ± 0.43^{b}	45
spring (Feb–Mar)	18	2.25–6.17	4.7 ± 0.32^{d}	33
summer (Apr–Aug)	16	1.04–5.11	3.4 ± 0.54^{fg}	18
total	72	1.04–9.76	4.92	30.5
Fresh fodder				
autumn (Sep–Oct)	17	0.76–3.87	2.9 ± 0.57^{g}	0
winter (Nov–Jan)	23	0.94–5.61	3.8 ± 0.41^{ef}	9
spring (Feb–Mar)	14	0.83–4.13	3.2 ± 0.27^{fg}	0
summer (Apr–Aug)	18	0.64–3.16	2.1 ± 0.64^{h}	0
total	72	0.64–4.61	3.04	2.8
Leftover bread samples				
autumn (Sep–Oct)	16	4.32–6.86	5.7 ± 0.83^{c}	75
winter (Nov–Jan)	22	6.42–11.34	8.9 ± 0.72^{a}	100
spring (Feb–Mar)	20	5.26–10.48	7.4 ± 0.68^{b}	100
summer (Apr–Aug)	14	3.96–7.86	4.9 ± 0.93^{d}	71
total	72	3.96–11.34	6.72	88.9

[a-g] – means with different superscripts within a column are significantly different at $P < 0.05$

AFB_1 in different feed types. The mean total content of AFB_1 in the commercial feed samples was estimated as 4.92 μg · kg^{-1}, however 30.5% commercial feed samples exceed the European Union (EU) permissible limit for AFB_1 in dairy feed samples, i.e. 5 μg · kg^{-1} (Martins et al., 2007). It was estimated by Kang'ethe and Lang'a (2009) that in Kenya 86% of tested commercial feeds for dairy animals AFB_1 contained AFB_1, and 67% of them exceeded the EU permissible limits. These results are much higher than those found in our study but clearly indicate the scope of the problem of contamination with AFB_1 and AFM_1. In Portugal, AFB_1 was found in 37.4% commercial feed samples (6.2% samples exceeded the permissible limits) (Martins et al., 2007). In a study conducted in Turkey, AFB_1 was found above the permissible limits only in two examined samples (1.9%) out of 104 samples of commercial feed mix for dairy animals (Koc et al., 2009). However in Iran, AFB_1 was found in all samples but at the permissible limits (Bagheri et al., 2014).

The reported levels of AFB_1 in commercial feed samples from Portugal, Turkey and Iran were much lower than those obtained in this study. Such differences might occur due to less favourable growth conditions for the fungus species responsible for the production of aflatoxins or due to more proper processing and storage conditions of dairy animal feeds. In line with our study, the elevated levels of AFB_1 from Pakistan in dairy animal feed samples were also reported by Chohan et al. (2016) and Ullah et al. (2016). The elevated levels of aflatoxins in animal feed samples might be associated with the lack of facilities to store dairy animal feed, favourable conditions for the growth and production of aflatoxins, illiteracy of dairy feed suppliers and insufficient monitoring by the government agencies (Miocinovic et al., 2017).

In the present study the mean level of AFB_1 in fresh fodder samples was 3.04 μg · kg^{-1}, and only 2.8% samples exceeded EU permissible level of this aflatoxin. Fresh fodder is not as prone to contamination as commercial feed due to the unfavourable environmental conditions for the growth of fungi in the fields. That is why animals fed fresh fodder have less chances for AFB_1 or AFM_1 poisoning. In Croatia, Bilandžić et al. (2014) measured the level of AFM_1 in cow's and goat milk samples: the mean levels of AFM_1 were 6.70 and 0.00 μg · kg^{-1}, respectively. The reduced level of AFM_1 in goat milk samples was connected with the fact that goats were fed fresh fodder, i.e. open grazing, whereas cows were fed stored fodder. Ashraf and Asif (2013) have also reported that chances of aflatoxin contamination of fresh fodder for dairy animals are lower in the rural areas of Pakistan since the availability of the fodder is better than in the cities where the stored fodder is used.

Maximum level of AFB_1 (mean value 6.72 $\mu g \cdot kg^{-1}$) in the present study was found in the leftover bread samples. Almost 89% leftover bread samples exceeded the maximum EU limit of contamination. Bread, traditionally made by mixing wheat flour with water, is the staple food for people in most of the countries around the world. In Pakistan it is a common practice that the leftover bread from homes and hotels is bought by street hawkers who redistribute it to dairy farmers. By the time the bread reaches the dairy farmers, it gets infested with mold. The contamination of bread fed to animals results in high level of AFM_1 and AFM_2 in the milk of animals (Ismail et al., 2016b). Moazenijula et al. (2007) studied the level of AFB_1 using the ELISA method in leftover bread samples used as fodder for dairy cows in Iran. In all samples the AFB_1 content above the permissible limit was found, also the milk from these animals was heavily contaminated with AFM_1 (above 0.05 $\mu g \cdot kg^{-1}$). Moreover, the bread contaminated with aflatoxins is also used as feed for pet animals and may cause serious disorders or even death, e.g., it was reported in Australia that many dogs were killed by aflatoxins present in the feed containing the leftover bread (Ketterer et al., 1975).

The presence of AFB_1 was confirmed in all feed samples. The level of contamination was the lowest in the fresh fodder – it means that dairy farmers should be encouraged to fed animals fresh fodder during the seasons of its availability. The elevated levels of AFB_1 in commercial feed samples indicate poor silage conditions which can be connected with humidity, temperature, and the activity of insects and rodents (Chohan et al., 2016). To prevent the contamination with aflatoxins leftover bread should be fed to animals immediately otherwise properly stored to prevent the grow of fungi and as a consequence the production of aflatoxins or simply discarded.

AFB_1 in different seasons. The temperature and humidity fluctuations are different during seasons. These two factors are closely connected with the growth of fungi as well as the production of aflatoxins (Sultana et al., 2013). A significant interaction ($P < 0.05$) was found between seasons and feed types. In all three dairy animal fodder types AFB_1 contamination can be described in the following order: winter > spring > autumn > summer (Table 1) – the highest in the winter and the lowest in the summer. The level of AFM_1 in milk is directly correlated with the level of AFB_1 in the feed for dairy animals. Similar effect of seasonal variation of AFM_1 level in milk samples from the same region was presented in our other study (Ismail et al., 2016b) and also by Ashraf and Asif (2013), and Aslam and Wynn (2015). Chohan et al. (2016) also found higher concentrations of AFB_1 in dairy feeds in winter (from December to March) than in other seasons. Mean maximum aflatoxin level in animal milk samples collected during winter season was also reported in other countries such as Iran (Fallah et al., 2016), Serbia (Tomašević et al., 2015) and Turkey (Golge, 2014). The elevated levels of AFB_1 in dairy animal feed samples during the winter season might be connected with the specific environmental conditions more favourable for the production of aflatoxins as well as the extended time of fodder storage.

Conclusions

Aflatoxin B_1 (AFB_1) is commonly present in the feed for dairy animals in Pakistan. Leftover bread samples were found to have alarming levels of AFB_1. Lack of awareness and less implementation of regulations are the critical factors responsible for the presence of aflatoxins in the feed for animals. Strict regulations and regular monitoring should be ensured to curtail the level of aflatoxins in animal feeds. Awareness seminars should also be organized for the dairy farmers about the health implications of aflatoxins in humans and animals.

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