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Original Paper

Aflatoxin contamination in Cotton Seed Cake used as dietary supplement in cattle in dairy farms of Sindh

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Abstract

The cotton seedcake is most widely used food supplement for dairy cattle in Sindh. Cotton seedcakes (CSCs) when not properly maintained under optimal conditions, lead to mold colonization, which causes aflatoxicosis in dairy farm animals. The aflatoxin (AFB_1) is transmitted through milk (AFM_1) and possibly pose great risk to human health. In the current study, prevalence and quantification of Aflatoxin in CSC has been carried out using the ELISA technique. Total 315 CSC samples were collected from dairy farms located in three zones of Sindh, i.e., the southern zone (districts Karachi, Thatta, Hyderabad), central zone (districts Mirpurkhas, Umerkot, Shaheed Benazir Abad) and northern zone (districts Naushahro Feroze, Sukkur, and Larkana). The findings suggest that AFB_1 levels were significantly ($p<0.05$) higher in the samples obtained from southern zone as compared to central and northern zone. Although, except Karachi (AFB_1 level 7.52 µg/kg), all other districts of all three zones were statistically non-significant, therefore, it is concluded that Karachi has high contamination of Aflatoxin contamination in CSC which may possibly cause the health risks in the population of area.

Keywords: Aflatoxin, Cotton seedcake, Prevalence, Quantification, AFM1.

Introduction

Aflatoxin is a toxin synthesized by a various fungal species of genus *Aspergillus*, which is distinguished by its bright yellow-green color. The name of toxin is derived from A= (Aspergillus) + Fla (flavus) + toxin. The aflatoxins B_1 and B_2, G_1, G_2 are synthesized by *Aspergillus flavus* and *Aspergillus parasiticus* respectively [1]. The toxin names B and G refer to the colors blue (B) and green (G). Under ultraviolet (UV) irradiation in thin layer liquid chromatography, the color of certain poisons tends to glow. These molds can penetrate food plants and are regularly observed on decaying or rotting material in maximum levels [2]. Drought stress, attack of insect, and lack of storage in hot places can also increase mold prevalence in food items. The four aflatoxins, known as B_1, B_2, G_1 and G_2, are the most dangerous of the 14 aflatoxins and are very hazardous for human and animal health. Various food sources such as nuts, grains, and their by-products are frequently attacked by *Aspergillus* producing aflatoxin and can be potential source to enter the food chain [3], [4].

Cotton, the main source of income in Southeast Asia, accounts for about 45 percent of the country's total oilseed crops. Pakistan's cotton seed production is expected to reach 9.178 million bales from 2020 to 2021 [5]. A machine-made product, cottonseed cake (CSC), is primarily used as a plant-based protein source for larger animals. Aflatoxin contamination of CSC causes significant annual losses to it. This is also a major problem for the cottonseed cake industry, as contaminated cottonseed meal tends to make animals sick and the aflatoxin is eventually excreted in the animal's milk [6], [7].

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The presence of aflatoxin in animal feed has been classified as an unwanted substance by the European Food Safety Authority [8]. Drought stress, high temperatures and insect infestation are all possible causes of aflatoxin contamination [9]. Aflatoxin M_1, the major mono-hydroxyl derivative, is generally considered to be the detoxification product of aflatoxin B_1, produced in the liver by cytochrome P450-related enzymes [10], [11]. Aflatoxin B_1 is rapidly absorbed from the gastrointestinal tract by an unannounced passive route [12] and passes into the milk [13], [14] through which the consumer becomes ill. The conditions that promote fungal growth not necessarily promote mycotoxin production. The external temperatures between 25°C and 30°C, water activity of 0.78 and above, and humidity of 88 to 95 percent are ideal for mycotoxin production [15].

The fluctuations in prevalence percentage between different regions are due to changes in climatic conditions around the world. Trichothecene is a mycotoxin which is common in temperate climates, while aflatoxin is much more common in tropical and subtropical climates. The contamination rates vary from year to year, with high prevalence in one year and low detection in the following year [16], [17]. Changes in the geographical range of mycotoxins type and production are thought to be responsible for climate variations. Most pre-harvest and post-harvest abatement techniques do not alter the stability of many mycotoxins, but instead lower their levels [18]. Other parameters that affect prevalence worldwide are pH, fungal strains, and substrate types [19]. Aflatoxin B_1 is a complete carcinogen and further chemical changes in it can cause tumors to develop into cancer during transformation and development [20]. Cottonseed cake was thought to cause a mysterious syndrome and the mortality in large ruminants under certain circumstances [21].

Aflatoxin-related symptoms in cows include liver and kidney damage, weight loss, and milk loss. Aflatoxin is degraded by many enzymes such as cytochrome P450 and glutathione S-transferase [22], [23]. To ensure food safety and quality, the detection and quantification of aflatoxin in food should be a major concern of food quality analysis. The contaminated milk, if consumed, can cause a significant health hazard for humans. Therefore, analysis and assessment of such contamination is of vital importance to ensure that animals are fed on healthy food supplements.

Since, limited evidences or studies on aflatoxicosis of cotton seedcake in Sindh, Pakistan, are available in literature, therefore, the current study was designed to monitor the prevalence and quantification of aflatoxin in cotton seedcake which most widely and commonly used food supplement for lactating cattle.

Materials and Methods

Sample collection and processing

The aim of this study was to determine the quantity of aflatoxin in cotton seedcake, which is commonly fed to commercial dairy cattle in various dairy farms as part of their daily diets.

The present study was carried out on cotton seedcake collected from different parts of Sindh, categorized into three geographical zones: Southern, Central and Northern during 2019-20. Three districts, with high density of dairy farms, were selected from each zone. Total 315 samples of cotton seedcake were collected from various dairy farms located in all three zones and transported to the Animal Products Technology Laboratory, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tandojam, in chilled (4°C) conditions. A commercially available ELISA kit (Bio-Shield Aflatoxin B_1 Extra Sensitive, Larissa, Greece) by Prognosis Biotech was being used to screen all of cotton seedcake samples for Aflatoxin B_1 detection and quantification.

Aflatoxin extraction and Analysis

The aflatoxin extraction from cottonseed cake was done using the Bio-Shield Total Extra Sensitive kit [24] following manufacturer instructions. A representative sample of cotton seed cake was ground to a fine instant coffee particle size (50 percent passes through a 20-mesh screen). Then extract was produced by mixing 20 g of powdered material with 80 mL of 70% methanol. The mixture was shacked for another 10-15 minutes. The sample-to-extraction-solvent ratio was kept fixed at 1:5 (w/v). After letting the solution mixture to settle for 2 to 3 minutes, 100 mL of phosphate buffer solution (PBS) was poured to it. To form a homogenate, the mixture was vortexed for 30 minutes. The 200 µl of the mixed aliquot were poured into dilution microwells and placed under dark at room temperature for 60 minutes. The 100 µl from each dilution microwells were transferred into the antibody coated microwells and incubated for 10 min at room temperature and washed for four times. Again 100 µl of detection solution was added and incubated for 5 min at room temperature. After that 100 µl of TMB substrate was added and left for 5 min at room temperature for the development of color and then 100

µl of stop solution was added in the last absorbance was noted at 450 nm within 60 min [24].

Statistical analysis

The statistical analyses were performed by using analysis of variance (ANOVA) in a computer-based application called Student Edition of Statistics (SXW), Version 8.1 (Analytical software-USA). The data were expressed as means± SD, as well as the least

Results

Prevalence of Aflatoxin B1 in CSC

Results for prevalence of AFB_1 in cottonseed cake are presented in Table 1. According to results out of 315 CSC samples collected from three zones of Sindh, about 238 (75.55%) were AFB_1 positive. The southern zone showed highest number of contaminated samples (80%), while northern zone showed lowest number of contaminated samples (69.52%).

Table 1. Zone-wise prevalence of aflatoxin in CSC.

Type of Aflatoxin	Prevalence (number of positive samples %)			
	Southern Zone	Central Zone	Northern Zone	Total
Aflatoxin B_1	84 (80.00%)	81 (81.14%)	73 (69.52)	238 (75.55%)

Aflatoxin B1 contamination levels in CSC

A. Southern Zone

Figure 1 shows the district wise profile of aflatoxin AFB_1 contamination in cottonseed cake samples collected from Karachi, Thatta, and Hyderabad districts. The average value in Karachi was highest (7.52 µg/kg), followed by Thatta (6.95 µg/kg), and Hyderabad with the lowest value at 6.58 µg/kg. Statistical analysis ($LSD_{(0.05)}$) showed that mean AFB_1 in Karachi district was significantly higher than Hyderabad but non-significant to Thatta. Since Karachi and Thatta are very near to Arabian Sea therefore, the prevailing winds are highly moisture loaded. This favors the growth of *Aspergillus flavus* on CSC. In result the higher levels of AFB_1 are produced in CSCs.

B. Central Zone

Figure 2 shows AFB_1 contamination levels in samples collected from three districts in central Sindh. According to results, the AFB_1 levels in the Mirpurkhas, Umerkot and Shaheed Benazir Abad were 6.67, 6.36, and 6.29µg /kg respectively.

C. Northern Zone

Figure 3 shows AFB_1 contamination of CSC in the samples collected from three districts of northern zone of Sindh. The results show that AFB_1 levels are 6.37, 6.25, and 6.08 µg/kg in the Naushahro Feroze, Sukkur, and Larkana respectively. Since all the districts show non-significant results therefore, it can be concluded that the AFB_1 levels found in these areas are statistically similar.

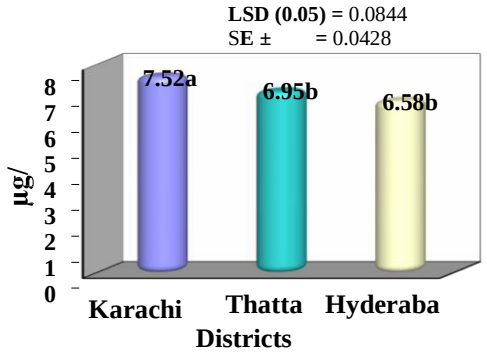

Figure 1. District wise Aflatoxin (AFB1) limits in Southern zone of Sindh.

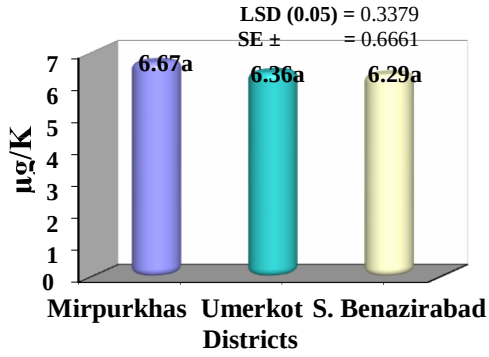

Figure 2. District wise Aflatoxin (AFB1) limits in Central zone of Sindh.

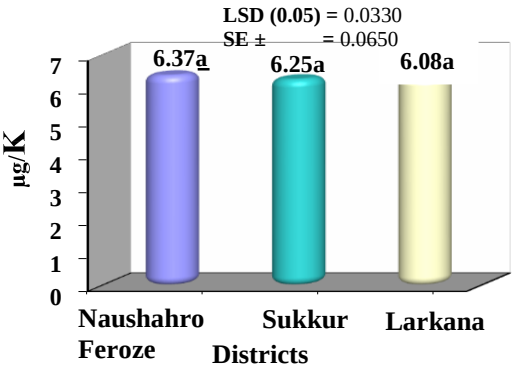

Figure 3. District wise Aflatoxin B_1 limits in Northern zone of Sindh.

Discussion

Cotton seedcake, also known as cotton seedcake meal, is a high-protein, energy, and fiber food for ruminants. The multifunctional cotton seed is covered by one of the finest human clothing materials, while it produces edible oil when pressed in oil mills, the remaining

pressed byproduct is cotton seedcake which serves as an important feed source for dairy cows. Cottonseed cake meals come in a variety of protein content percentages. It has a high fiber content in terms of percentage which is quite palatable. Storage proteins in the presence of carbohydrates during developing cottonseed cake meal can promote Aflatoxin activation [2]. When ruminant animals ingest contaminated feed containing Aflatoxin B_1, the contaminant is transformed into aflatoxin M_1 by the cytochrome P450 oxidase system, which is found in the rumen microflora of animals and associated cells. Aflatoxin M_1 secreted in milk can be equivalent to the 3 percent of aflatoxin B_1 ingested by the animal [25]. Aflatoxins are hazardous to humans and animals both acutely and chronically, causing liver damage, cirrhosis, tumor induction, and teratogenic consequences [4].

In this study, the observed CSC samples had Aflatoxin B_1 concentrations below the European Commission's recommended maximum residue limit (MRL), which itself is 20 µg/kg. Based on the AFM_1 value of milk, the concentration of aflatoxin B_1 in dairy cattle feed should be 1.96 µg/kg [26]. Our studies confirm the findings of [27] who reported 80 percent of cottonseed cake samples in Pakistan contaminated with aflatoxin B_1 however, they reported an average contamination level of 69 g/kg which is far low from the findings reported in this study. Their reported values are higher perhaps due to presence of Aflatoxin AFB_2, AFG_1, and AFG_2 as coupled with aflatoxin B_1 in the same samples.Amanullah et al., [28] explored Aflatoxin B_1 in cotton seed cake, Wanda, wheat bran, and homemade concentrate mixture of dairy goats in Pakistan and found an overall prevalence of AFB_1 83% samples, which also confirm our findings. However, their levels of contamination ranged from 0 to 225.736 ppb in different feed samples, with the highest possible level of aflatoxin B_1 detected in cotton seed cake (mean level 137.059 ± 22.293 ppb).

The occurrence of microbial contamination is influenced by changes in environmental conditions and agricultural activities patterns. Fungal growth and mycotoxin synthesis are enhanced by high temperatures and humidity. It was observed that the southern zone had the highest percent of AFB_1 in CSC (80 percent). The higher contamination in southern zone may be due to high levels of humidity in air where the CSC bags are stored. Because southern region of Sindh is near to Arabian Sea from where moisture laden winds carry moisture and increase aerial humidity in these regions. Opposite to this the northern region remains hot and dry most of the time of year having very low humidity levels in air. These conditions does not favor the growth of *Aspergillus flavus* on CSC. *A. flavus* and *A. parasiticus* can survive in feeds with moisture levels of 13 to 18 percent and ambient moisture levels of 50 to 60 percent. Changes in aflatoxins prevalence in the current study could be related to high humidity in the southern zone due to costal (Karachi and Thatta) and highly irrigated areas (Hyderabad), followed by heavy low humidity in the northern zone (upper Sindh). The southern area of Sindh province is hot but usually humid due to the closeness of the sea, particularly contrast to the central and northern areas, which are hot but less humid.

From Hyderabad and Mirpurkhas, the incidence of AFB_1 in cottonseed cake was found high (82.86 percent). The high percentages could be linked to humidity and large-scale commercial dairy production in these districts compared to the rest of the study areas. The cultivation of fodder is being reduced day by day due to rapid urbanization, especially in Karachi and Hyderabad, and severe salinity in the Thatta district. Cottonseed cake is in great demand because of limited cultivation of fodder crops to meet the needs of commercial dairy farms. In Karachi and Thatta, there are no cottonseed cake producing mills, therefore, cottonseed cake and other grasses are carried from Sindh's central and northern parts therefore are stored for long periods of time. The cottonseed cake is purchased in bulk and stocked due to price fluctuations, but the storage conditions are not with sufficient aeration/ ventilation, hence the aflatoxins develop quickly and at high rates [29], [30], [3], [31]. The CSC may become susceptible to aflatoxin B_1 infestation because of improper aeration during transportation from one to other region. Environmental factors particularly pre- and post-harvesting conditions of the crops (mainly soil condition and kind of seed sowed, pest attacks, and so on) have been considered as key reasons of Aflatoxin level variation [2], [32]. Therefore, findings of this study were with that of the [33], who found that cottonseed cake alone was responsible for 80 percent of total aflatoxin intake in dairy animals due to the high contamination with mycotoxin and quantity in the milk producing animal ration.

Conclusion

Aflatoxin in cotton seedcake was tested and quantified. From the observations it is clear that the amount of AFB_1 was below the EU allowable limit in all zones of Sindh province. In Karachi district, AFB_1 levels were higher and cotton meal samples from Larkana district seem to have lower levels of AFB_1.

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Conflict of Interest

There are no conflicts of interests in publishing this manuscript.

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