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Quantitative Detection of Aflatoxin M1, Ochratoxin and Zearalenone in Fresh Raw Milk of Cow, Buffalo, Sheep and Goat by UPLC XEVO -TQ in Dakahlia Governorate, Egypt

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Research Article

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Abstract

Animals' derived food as milk is highly nutritive food for human and substitute mother's milk for children especially after 6 months. To better produce and maintain safe milk and milk product, Ultra Performance Liquid Chromatography XEVO-TQ (UPLC) used to monitor mycotoxins as aflatoxin, ochratoxin and zearalenone levels in fresh raw milk of dairy domestic animals. AFL-M1 was detected in all samples in cow, buffalo, sheep and goat milk with different concentration ranged from high, medium and very low with mean 906, 811.1, 1394.86 and 1183.68ng/L respectively. According worldwide standard for aflatoxin M1 level in milk as EC and according US FDA Limit, all samples of raw milk exceed this limit up to 100ng /l while 50%, 80, 90 and 60% exceed US FDA Limit. Notably, the Egyptian raw milk is free from ochratoxin and zearalenone mycotoxin. Taken collectively, strict strategies should be taken to reduce level of aflatoxin in raw milk between producer and consumer and so reduce risk of adverse effect on health.

Keywords: Aflatoxin M1; Ochratoxin; Zearalenone; Fresh Raw Milk

Introduction

Consumption of contaminated food leads to acute or chronic toxicity in animals and human, which accompanied by many adverse effects on the cardiovascular, central nervous, gastrointestinal and pulmonary systems and may be carcinogenic, teratogenic and immunosuppressive effects [1]. There is also an eminent hazard on human health from the ingestion of animal derived foods such as milk which have mycotoxins residues or their metabolites [2]. Aflatoxins are classified as food contaminant and some of their metabolites enhanced genotoxicity and carcinogenic potential. Therefore, understanding the hazardous effect of mycotoxins on human, has led to the necessity of

development of new strategies to reduce the aflatoxins exposure for human [3,4].

Aflatoxins are exo-secreted metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus* which can be found in a wide range of food and feed. Due to the known harmful potential of these toxins to human and animals, legal limits have been set in many countries. European Commission (EC) has established a maximum acceptable level of aflatoxin M1 (AFM1) in milk to be 50ng/L [5,6]. The contaminated feed is the main source for mycotoxins infestation of farm animals. Oral intake of fungal metabolites with feed results in a negative impact on all relevant parameters of animal production. Moreover, under experimental conditions, mycotoxins and/or their metabolites can be traced in milk [7]. Additionally, Ellis, et al. [8] estimated that factors such as season, time consumption and improper handling of food can be involved in the presence of AFM1 in milk. In addition, the amount of AFM1 in the rainy season is greater than the dry season. Aflatoxin M1 can be found in animal milk within 12-24h after the first ingestion of aflatoxin B1 (AFB1) and can last up to 3 days after the last ingestion of the mycotoxin. The rate between the amount of AFB1 ingested by the animal and the quanitity excreted in milk is usually 0.2 to 4% (Henry et al., 2001).

Ochratoxin A (OTA) is a secondary metabolite produced by many species of *Aspergillus* and *Penicillium* fungi. OTA is also one of the harmful mycotoxins that posed a threat to animal and human health [9-11]. Notably, dietary exposure to OTA may have association with endemic nephropathy, a chronic progressive hypercreatinemia, uremia, hypertension and oedema and considered carcinogenic to human [12,13]. Zearalenone (ZEA) is a secondary metabolite secreted by several species of *Fusarium* fungi, mainly *F. graminearum* and *F. culmorum.* These species are known to grow on maize, barley, oats, wheat and sorghum. Both human and animal exposure comes from chronic ingestion of contaminated-food. In addition, human exposure can be direct via cereals or indirect via animal products [14]. ZEA and its metabolites can be excreted into milk, but levels are very low, and often remain below the limit of quantification [15]. ZEA has a pronounced estrogenic action and numbers of animals are susceptible to it. It has been stated that zearalenone may stimulate growth of cells with estrogenic receptors in human mammary gland. It is so supposed that zearalenone may lead to breast cancer in human being.

In the view of aforementioned hazardous effects of these toxins, the current study was planned to evaluate the occurrence and concentration of mycotoxins in fresh raw milk of different species at Dakahlia Governorate, Egypt.

Materials and Methods

Collection of the Samples

A total of 120 raw milk samples, (30 each of cow, buffalo, sheep and goat) were randomly collected from Aga City, Dakahlia Governorate, Egypt at winter season, 2017. Each sample was subjected to aflatoxin, ochratoxin and zearalenone analysis using Ultra Performance Liquid Chromatography XEVO-TQ (UPLC). The collected samples were transferred to laboratory in clean, dryand tightly closed bottles and kept in ice box.

Extraction of Mycotoxins from Raw Milk

All chemicals used were of HPLC grade (Sigma Aldrich, Cairo). Extraction of mycotoxins was done by disposable column c18 which was cleaned firstly by 10 ml of methanol followed by 10 ml of milli-Q water. 20 ml of fresh raw milk diluted with 30 ml of Milli-Q water was loaded into the column. After loading, the column was washed with 10 ml of Milli-Q water and dried by 10 ml hexane. The column c18 was then dried before elution [16]. 8 ml of Acetonitrile (ACN) was added to extract mycotoxins according to the method described by Huang, et al. [17]. The whole mixture was vortexed for 2 min by using Vortex-Genie 2 and then kept in an ultrasonic bath for 30 min. Finally, the mycotoxin extracts were centrifuged with cooling at 4°C, 12,000 RPM for 10 min and the supernatant was separated. The total supernatant was concentrated until 2 ml by evaporation at 50 °C under a nitrogen stream. The concentrated supernatant was mixed with 4 ml of milli-Q water and the pH was adjusted up to 5.0 ± 0.2.

Preparations of standard combined stock solution: The standard stock solutions of (300, 500 and 700 ng/L) of each AFM1, OTA and ZEA (Sigma Aldrich-Cairo) were prepared in methanol. The combined standard solution was prepared by taking 1 ml of each stock solution of 300, 500 and 700 ng/L of AFM, OTA and ZEA to 97 ml of methanol/water (50/50 v/v) in a volumetric flask, followed by further dilution with high purity methanol up to a final volume of 100 ml. Each individual mycotoxin stock and the combined mixtures of standard solutions were stored at -20°C before use.

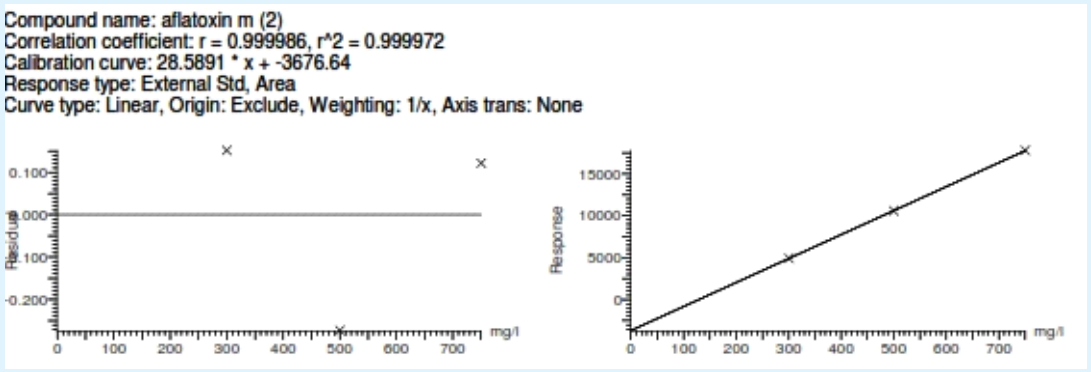

Figure 1: Graphical representation of standard combined stock solution.

Quantitative Detection of Mycotoxins Level By Ultra Performance Liquid Chromatography (Acquity Uplc - Xevo Tq Ms System):

- Equipment: ACQUITY UPLC - Xevo TQ MS system (MS/MS Detector) (UPLC XEVO apparatus modules):
- Quaternary high-pressure pump with 1500 psi (Waters Company USA).
- Auto sampler, Wither cooling sensor (Waters Company USA).
- Column manger (Waters Company USA).
- Software Math lynx for data analysis (Waters company USA).
- MS/MS XEVO with Photomultiplier detector.

The Mobile Phase was a Mixture of

- 2mm ammonium acetate solution.
- 0.1% formic acid.
- Water.
- Methanol HPLC Grade.

UPLC Columns

- Packed with very fine particles (stationary phase)
- The separation was done hypersil gold™ C18 (1.7µm, 2.1×100mm), HLB Waters company, with mobile phase as described and flow rate was 0.4 ml/min.
- Syringe filter: PTF membrane pore size 0.45mm.

Chemicals

- Methanol (HPLC grade, Sigma Aldrich, Cairo).
- Acetonitrile (HPLC grade, Sigma Aldrich, Cairo).
- Formic acid (85%) (Pure reagent for analysis, EL-Nasser Pharmaceutical Chemicals Company prepared as 0.1%.
- Ammonium acetate solution.

Chromatographic Conditions

Mobile phase (A) of 2 mM ammonium acetate with 0.1% formic acid in water.

mobile phase (B) of 2 mM ammonium acetate with 0.1% formic acid in methanol with flow rate 0.4 ml/min, injection volume 50µl with mix gradient elution of two mobile phase.

Time (min)	A	B
0	90%	10%
3	90%	10%
10	30%	70%
10.1	10%	90%
12	10%	90%
12.1	90%	10%

Table 1: Chromatographic Conditions.

Gradient

- Detection was done with Xevo MS/MS with Photomultiplier detector. While, the quantification of residues in sample was obtained and calculated from area under curve, extrapolated automatically by software (math lynx).
- MS Conditions
- MS System: Acquity Xevo Tp
- Ionization mode: ESI+
- Desolvation Temp: 600 °c
- Capillary Voltage: 0.8 KV

Statistical Analysis

- One-way ANOVA was adopted in this study. Mean ± standard error of mean (SEM) and descriptive analytical parameters were calculated [18]. Computerized SPSS program version 13 was used in the evaluation of data.

Results and Discussion

Safety of animal derived food as milk should monitor from time to time to ensure freedom form mycotoxins or compliance with the established standard limit in by Egyptian regulations and different organizations and countries.

The range and levels of AFM1 concentration in samples were given in table 2 and figure 2 with highest mean concentration of all samples recorded in sheep>goat>cow>buffalo (Table 2). Notably, AFM1 levels were varied from samples from one area to another in same species and also in different dairy animal's milk (Figure 3).

Dairy species	No.	Positive samples		Mean ± SE	95 % Confidence Interval		Minimum (ng/L)	Maximum (ng/L)	P value
		No.	%						
Cow	30	30	100	1609.69±460.43	668.01	2551.37	146.1	8547.7	> 0.05^{NS}
Buffalo	30	12	40	1187.06±360.02	394.65	1979.47	187.3	4646.8	
Sheep	30	10	33.33	2032.63±673.75	508.51	3556.75	531.6	7776.1	
Goat	30	8	26.67	1030.48±364.88	167.67	1893.28	139.5	2715.7	

Table 2: Occurrence of AFM1 in raw cow, buffalo, sheep and goat milk samples (N=30 each).
One- way ANOVA was run to determine differences between four species of animals (cow, buffalo sheep, and goat) at level of aflatoxin M1.Test result indicated that there was no statistical difference between the four species.
F at (3,36)=1.003 P > 0.05.
N= number; SE= standard error; NS= Non-Significant.

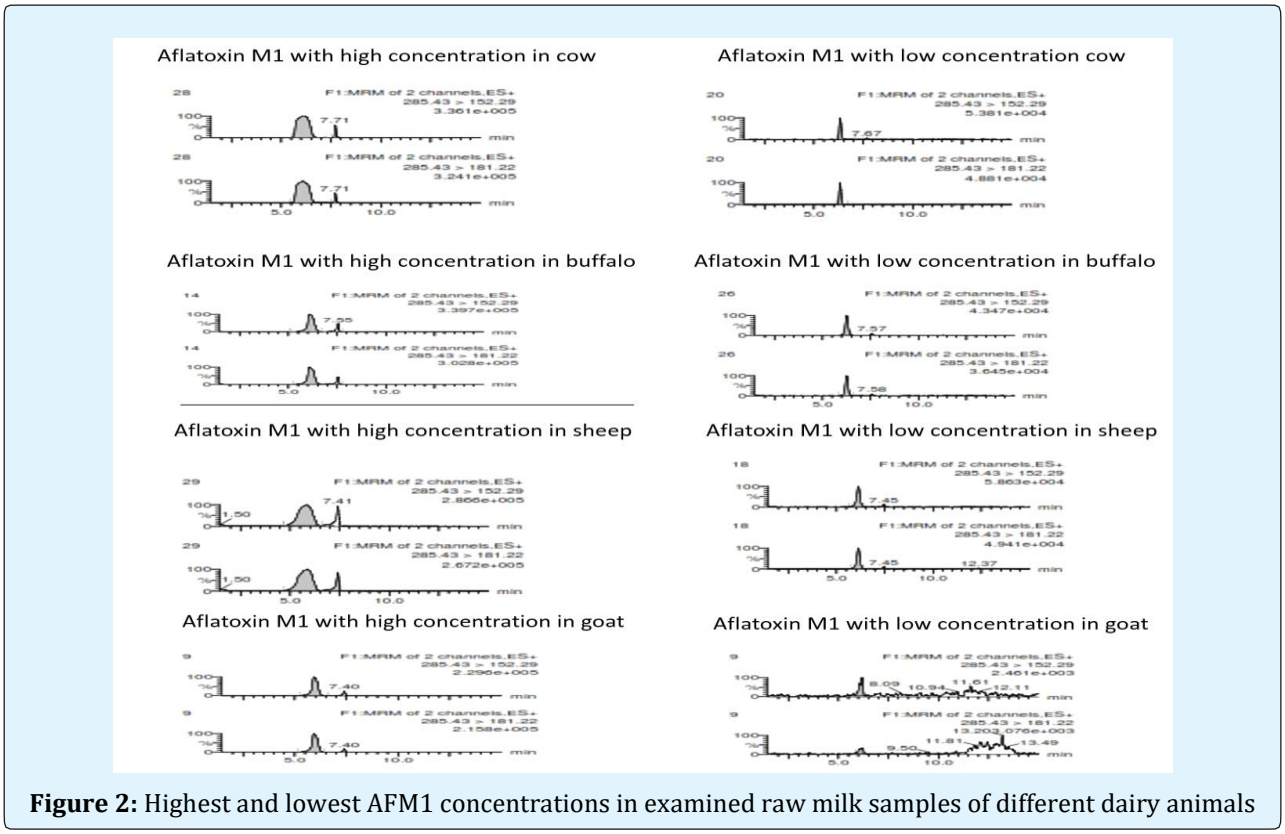

Figure 2: Highest and lowest AFM1 concentrations in examined raw milk samples of different dairy animals

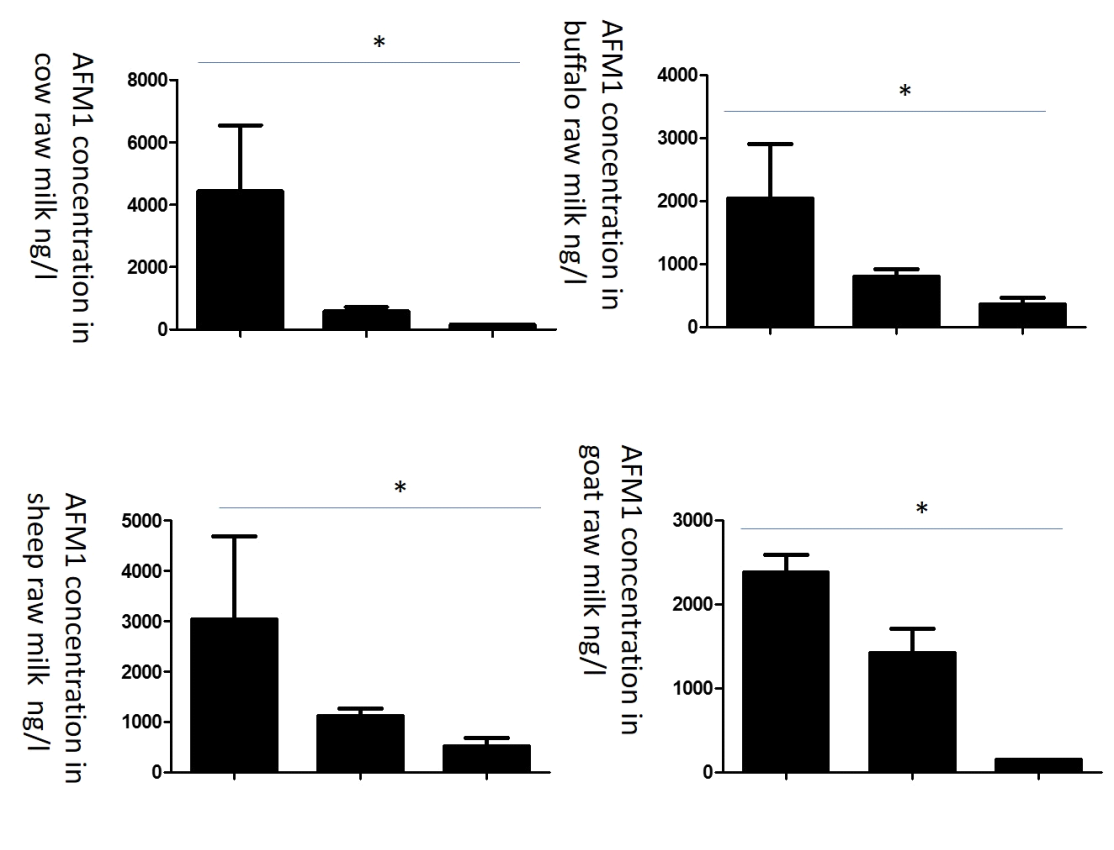

Figure 3: Show high, medium and low concentration of aflatoxin M1 in different dairy animals. Notably, AFM1 high level recoded more in cow >sheep>goat>buffalo while low level noticed in goat milk.

According worldwide standard as ER, EC, all samples of raw milk exceed this limit up to 140 ng/l and also most samples exceed US standard (Table 3).

Raw milk	Positive samples		Exceeding ER Limit (50 ng/L)		Exceeding EC Limit (50 ng/L)		Exceeding US FDA Limit (500 ng/L)	
	N	%	N	%	N	%	N	%
Cow	30	100	30	100	30	100	15	50
Buffalo	30	100	30	100	30	100	24	80
Sheep	30	100	30	100	30	100	27	90
Goat	30	100	30	100	30	100	18	60

EC: European Commission, (2006), the limit in milk is 50 ng/L. US FDA: US FDA, (2011) the limit in milk is 500 ng/L. ER: Egyptian regulations, (1990), the limit in milk is 0 ng/L.

Table 3: Levels of AFM1 (ng/L) in raw cow, buffalo, sheep and goat milk samples exceeding limits established by Egyptian regulations, the EC/Codex and US FDA.

Notably, Egyptian raw milk is free form ochratoxin and zeralenone mycotoxins (Tables 4 & 5).

Dairy Species	Examined samples	Positive samples	Negative samples
Cow	30	0 (0.00%)	30 (100%)
Buffalo	30	0 (0.00%)	30 (100%)
Sheep	30	0 (0.00%)	30 (100%)
Goat	30	0 (0.00%)	30 (100%)

Table 4: Occurrence of Ochratoxin in raw cow, buffalo, sheep and goat milk samples collected locally from Dakahlia governorate, Egypt.

Dairy Species	Examined samples	Positive samples	Negative samples
Cow	30	0 (0.00%)	30 (100%)
Buffalo	30	0 (0.00%)	30 (100%)
Sheep	30	0 (0.00%)	30 (100%)
Goat	30	0 (0.00%)	30 (100%)

Table 5: Occurrence of Zearalenone in raw cow, buffalo, sheep and goat milk samples collected locally from Dakahlia governorate Egypt

Discussion

There is increase concerning for production Egyptian raw milk free from mycotoxin as milk consumed in large scale with population. There is a different standard used in Egypt compared with other countries as ER, EC and US. [19] Conducted that maximum residue limit for AFM1 in raw milk is zero for Egyptian regulation, 0.05µg\L according to EC, while maximum residue limit in the US for AFM1 is 0.5µg\L for milk [20]. Also it found that an acceptable level of risk for AFM1 in fresh raw milk at 0.05 µg kg^{-1} according to regulation of the Codex Alimentarius and Joint Expert Committee on Food Additives (JECFA).

The present study found that all samples under experiment have an aflatoxin M1 with different levels from high, medium and low concentration in all species with 50% for cow, 80% buffalo, 90% sheep and 60% goat milk Exceeding US FDA Limit (500 ng/L)US. As recorded and determined in different report by Anonymous [6] who established that the maximum acceptable level of AFM1 in milk is 50 ng\L by the European Commission (EC), Bakirci [21] who examined 90 raw milk samples for AFM1 and found that 79 (87.77%) were aflatoxin M1 contaminated and 35 (44.30%) of the positive samples were higher than the maximum tolerance limit (0.05 ppb) accepted by Turkey and European union (EU) and El-Sayed, et al. [22] who examined 15 Egyptian cow's milk samples and found that 3 out of them were positive for AFM1 with a mean value of 6.3 ppb.

Notably, similar study to our finding recorded that all 177 fresh milk samples except one from Kuwaiti markets were contaminated with aflatoxin M1 ranging from 4.9 to 68.7 ng/kg, but 8 samples exceeded EC regulatory limit [23]. Additionally, Sadeghi, et al. [24] examined 320 raw milk samples and revealed that all the samples had measurable contents of AFM1 According to Codex standard, 25 samples (7.82%) of all the samples had aflatoxin content lower than the permissible content of the Codex standard, 295 samples (92.18%) contained aflatoxin M1 exceeding the permissible contents of the Codex standard. Out of 320 samples 295 (92.18%) had more than 0.5 µg/L of aflatoxin M1. In contrast, Li, et al. [25] found that only 1.1% of raw milk samples exceeded the EU standard limit (50 ng/L), and none of all samples exceeded the Chinese and United States legal limit (500 ng/L). This explains that strategies control approved by each country could able to reduce the limit of aflatoxin in milk and reduce risk effect of mycotoxin in the health of human.

The current study found that raw milk free from ochratoxin as described before Skaug [26] no ochratoxin A was detected in any of the 20-prepared infant formula of cow milk samples detected by means of HPLC method or in Germany cow's milk samples or in Egyptian raw milk in El-Minufia governorate [27,28]. Also Bascarán, et al. [29] and Moudgil, et al. [30] found that no OTA was detected over the established limit 0.5 ng/ml and is below maximum residue limit of 2ng/ml. Notably, Gonzalez-Osnaya, et al. [31] summarized that no information is available on the rate of transfer of OTA into milk of dairy cows; OTA concentration in bovine milk are usually low, although some exceptions may occur if cow are ingesting large amount of OTA. Even though only low concentration of OTA may be present in milk, these small amounts may be important to consumers of large quantities of milk, particularly children.

The presented study reported that no zearalenone residue in fresh raw milk as Seeling, et al. [15] who conducted that zearalenone mycotoxin and its metabolites can be found in the milk, but levels consider very low and often remaining below the limit standard level. Also, Kalac [32] mentioned that when the animal feed supplemented ration with silage, the main mycotoxin contaminated are DON and ZEA. These contamination contents can be decreased by the rumen microbiota in healthy animals, thus reducing the risk of milk contamination. While Mahmoudi [33] found Zearalenone in 15 (21.42%) of raw milk samples with mean level 1.34+-1.42 ng/ml.

In the current study we found that AFM1 was found in all samples but with differ levels ranged from high, medium and low in raw milk and also in different dairy species. Even low level still exceeds than Egyptian regulation that mention that AFM1 should be at zero level. Similar record [31] explains high level of AFM1 in raw milk due to composition of diet of dairy animals is mainly silage or contaminated feed stuff. Additionally, it was found seasonal effect on AFM1 level in summer lower than winter or due to distribution effect as far distance between producer and consumer. While low concentration of AFM1 in raw milk in some samples was explained due to mixing and dilution of contaminated one with other which had little or may be non-contaminated one from different resources. While there was no effect of storage, processing and manufacture in level of AFM1.

On conclusion, strategies control approved by each country could able to reduce the limit of aflatoxin in milk and reduce risk the effect of mycotoxin in the health of human. So regulation in Egypt according quality assurance should have applied to minimize the level of mycotoxins and its adverse effects.

Conflict of Interest

There is no conflict of interest of all authors

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