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SEASONAL MONITORING OF AFLATOXIN M1 IN ROW MILK SAMPLES AT SOHAG GOVERNORATE

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ABSTRACT

This work monitored the aflatoxin M1 (AFM1) in raw buffalo milk trailed in Sohag city, Egypt in different seasons. Milk samples were obtained from March to October 2021. The enzyme-linked immunosorbent assay (ELISA) was used as a methodology technique. The results of AFM1 presented that the highest frequency of occurrence, with a detection incidence of 85.5 % in winter samples and 78.5% in summer samples. The positive samples showed range levels of AFM1 between 0.06 to 0.8μg/kg in winter samples and range of 0.0 to 0.9 μg/kg in summer milk samples. The percentage of aflatoxin M1 samples exceeded the MRL of Egyptian Standard Regulation 2010/7136 last updated, are 78 and 100% in two examined seasons, respectively**.** The residue levels of AFM1 obtained in the investigated samples; represented a serious concern about the health risk of consumers. It is worthy to sit a regular schedule for monitoring and inspection of dairy products for aflatoxin residues.

Keywords: Aflatoxin M1, row buffalo milk, enzyme-linked immunosorbent assay

INTRODUCTION

Mycotoxins are secondary metabolites provided basically by fungi group related to the *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Claviceps* genera. These fungi can grow on deferment crops in agriculture and forming mycotoxins through pre- and post-harvest times, processing, and storage (Ogunade *et al.,* 2018). When animals are given feed contaminated with mycotoxins, these toxic compounds are metabolized and passed to animal tissues and polluted the food derived from animals such dairy products including row milk milk (Negash, 2018).

Aflatoxins are polycyclic compounds related to the furanocoumarin group of structures (Williams *et al.,* 2004) fungi metabolites basically formed by *Aspergillus flavus* and *parasiticus* (Prandini A, Tansini G, Sigolo S, Filippi L, Laporta M, Piva G. 2009). Mycotoxins may cause different hazards in animals and humans, including carcinogenicity, mutagenicity, teratogenicity, immunotoxicity, and estrogenic effects (Gallo *et al.,* 2015). When dairy cattle are given feed contaminated with AFB1 residues, the consumed mycotoxin is bio-transformed by liver enzymes into

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different metabolites. The hydroxylation process of tertiary carbon converted the di-furanocoumarin ring of AFB1 into the main metabolite, AFM1 (Assaf *et al.,* 2019).

Animals fed on rations contaminated with AFB1 excreted through milk multiple residues of major hepatic 4-hydroxylated metabolite called as "milk toxin" or AFM1. Razing the lactating cattle on polluted maize resulting in huge milk contamination with AFM1 (Pietri *et al.,* 2009). AFM1 resists the thermal treatment and the pasteurization and sterilization methods are not satisfied for the inactivation (Assem *et al.,* 2011).

Variable criteria influenced the presence of the fungi Aspergillus genera and affect the incidence of contamination by aflatoxin. The pollution may be existed at any step of harvesting pre or post, and in different stages food processing or storage. The major element can affect aflatoxin production is related principally to the climatic change of the region, the type of the plant, soil category, the daily temperatures, and the moisture content (Serraino *et al.,* 2019). Aflatoxin formulation is also stimulated with harvest -stress and crop damage which due to drought and many stresses in pre- harvest, insect flaring up, incorrect schedule of harvest. Humid weather, rising in temperature, and poor aeration through storage are also detrimental factors (Madali *et al.,* 2018).

The existence of AFM1 in dairy products, even in small amounts, indicates a huge concern, especially due to these products are mainly ingested by children who are more sensitive category to the aflatoxins hazards and their insufficiency of metabolism and immune function (Kunter *et al.,* 2017; Fakhri *et al.,* 2019).

AFM1 represents the main metabolite can induce carcinogenicity in milk, and is closely resemble in its acute toxicity to AFB1. Therefore, AFM1 is classified as a class 1 human carcinogen (IARC, 2012). Chronic low-levels exposure to aflatoxin is resulted in enhance the risk of hepatic carcinoma in human, and leading to suppression of immune function and has a great act in childhood stunting (Muaz *et al.,* 2022). Therefore, most countries have developed legal residue limits of AFM1 in milk, ranging from the 50 ng/L set by the European Commission Regulations (165/2010) (EU, 2010), and to the 500 ng/L developed by US Food and Drug Administration (US FDA, 2011). However, Egypt minstrel of health has recommended on a zero-tolerance strategy in milk and dairy products (EOSQC, 1990; Amer and Ibrahim, 2010). However, this goal (0 ng/L) will be impractical to reach as long as aflatoxin residues contamination in derived feed from animal origin. In this regards, there is last update of Egyptian Regulation, 2010/7136 (ER, (2010) set the MRL at 50 ng/L of AFM1in liquid and row milk. Consequently, the goal of the present work was to determine the incidence and concentrations of AFM1 in raw buffalo milk samples obtained from two different seasons winter and summer, where the samples produced locally and consumed in Sohag governorate. In addition, it was to compare our findings against the several regulations regarding AFM1 that have been legislated by the EU, US FDA, and Egyptian Regulations.

MATERIALS AND METHODS

[I] Chemicals and reagents:

- Acetonitrile for sample preparation.
- Chemicals and reagents were obtained from aflatoxin M1 Elisa 96t, SINOGENECLON CO., Ltd (china).

- They included the microcelisastip plate 8 well x12 strips and different standard concentrations at 0, 0.05, 0.15, 0.45, 1.35, 4.05 ppb (black cap) 1 x 1.0 ml,
- Antibody working solution (blue cap)1x5.5 ml,
- enzyme conjugate (red cap) 1 x5.5 ml ,
- sub A, B sol 1 x 6 ml ,1 x 6 ml ,
- stop sol. reaction 1 x 6 ml ,
- solution of wash 20x 1x40 ml,

- re-dissolving conc. sol 2x, 1x50 ml,

[II] Samples and sampling:
A total of twenty-eight samples (500 ml per sample) of whole buffalo raw milk were obtained randomly during the winter and summer seasons (14 samples in the winter and 14 samples in the summer) in the period between March and October 2021 from different locations of whole buffalo raw milk production at Sohag city. The collected samples were placed into sterile plastic bottles identified and transferred to the Central Laboratory of Toxicology Department of the Veterinary School, Assuit University, under refrigerated conditions in an ice box and kept under 4°C until further analysis (Table 1).

Table 1: Experimental design of the study at Sohag governorate in different two seasons.

Season	Period	Number of samples
Winter	(12/3/2021 to 20/4/2021)	14 sample
Summer	(22/6/2021 to 21/9/2021)	14 sample
Total number of samples	----------	28 samples

Preparation of milk samples:
For all types of milk samples, 1 ml of milk samples were added into 50ml centrifuge tube, pipette 4 ml acetonitrile were aspirated, mixed for 5 min and make centrifugation at 4000 RPM for 10 min. 2.5 ml were aspirated supernatant and let dry at 50 to 60 °C with dry water bath adding 1 ml re-dissolving solution (Liquor 2), oscillate fully. 50µ were taken out for test and this solution was applied directly to ELISA microtiter plate.

Reagent Preparation
Liquor 1 (Sample extracts solution): 84% acetonitrile - water solution. V (Acetonitrile). V (Deionized water) = V21: V4

Liquor 2 (Redissolving solution): 2 times dilution then 2x concentration with deionized water.

Liquor 3 (Washing buffer): Dilution of 40 ml of the concentrated washing buffer (20 x concentrated) with the distilled water to 800 ml.

ELISA test steps:
According the manufacture's kit steps, adjust the absorbance reading at wave-length 450 / 630nm), within 10 min.

The results interpretation
The calculation of the percentage of absorbance value and the final concentration obtained from standard curve Fig.1.

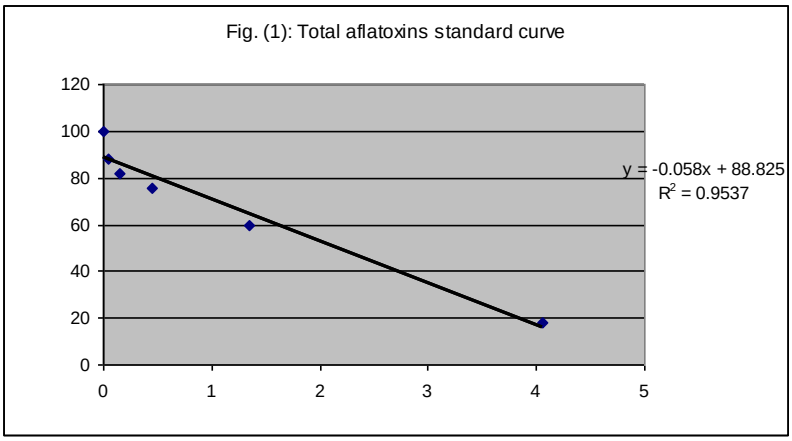


Fig. 1: Calibration curve of AFM1 standards

Statistical analysis

- Results were recorded as mean ± SD. Percentage, minimum, maximum and mean ± SD were obtained. Significant analysis performed using the one-way ANOVA with a significance level at $P < 0.05$.

RESULTS

Occurrence of aflatoxin M1

- In this study, the examined level of aflatoxin M1 showed range of concentration between 0.0 - and 0.9 µg/kg with a mean value of 0.48± **0**.31 µg/kg for winter milk samples. The examined level of aflatoxin M1 in summer milk samples showed range levels between 0.06 - 0.8µg/kg with a mean level of 0.23±0.30 µg/kg, Table 2.

Table 2: Levels of Aflatoxins M1 (µg/Kg) in milk samples at different seasons.

Sample	No of sample	Aflatoxins M1 in positive samples	
		Concentration range (µg/Kg)	Mean concentration +SD (µg/Kg)
Winter	N=14	0.0 - 0.9	0.48± **0**.31
Summer	N=14	0.06 - 0.8	0.23±0.30

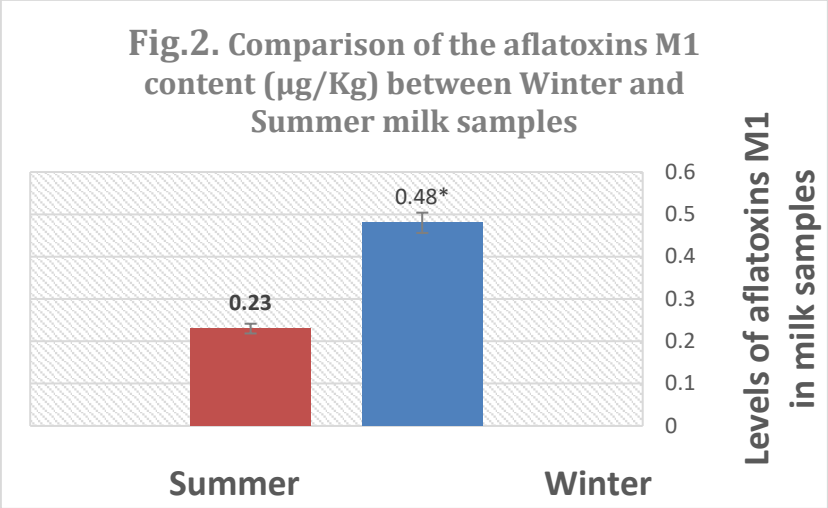
The comparison of aflatoxins M1 levels in different seasons

- The average values of the examined aflatoxin M1 of showed a significant difference at $P < 0.05$ between the winter and summer milk samples (Table3 and Fig 2).

Table 3: Comparison of the aflatoxins M1 content (µg/Kg) between different seasons of a year in raw milk samples consumed in Sohag Governorate.

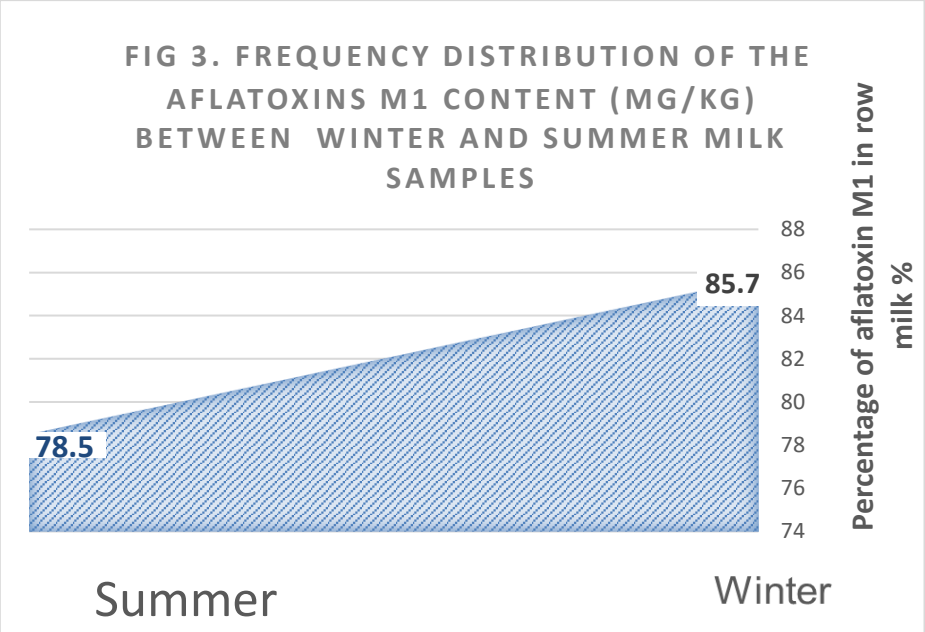
Samples in different seasons	Range levels (µg/kg)	Mean + SD (µg/kg)	*P value* < 0.05
Winter	≤0.9	0.48+ **0**.31	*
Summer	≤0.8	0.23+0.30	*

***** *Significant value 0f M1 between winter and Summer n=14.*



The frequency distribution detection

- The percentage of presence was 85.5 and 78.5 % for aflatoxin M1 in winter and summer milk samples, respectively, as showed in Fig 3.



The comparison with the international MRLs

- The percentages of aflatoxin M1 samples exceeding MRL of European Union (Eu) (2006), are 78 and 100 % in the examined winter and summer milk samples, respectively, where the limit for AFM1 MRL is 0.05 µg/kg. In comparison with the MRL of Codex Alimentarius (FDA/WHO) (2001), the percentage of aflatoxin M1 samples exceeding the limit are 78 and 100 % in winter and summer milk samples, respectively, where, AFM1 MRL is 0.05 µg/kg. The percentage of aflatoxin M1 samples exceeded the MRL of the US FDA, (2011) limit for AFM1 are 50 and 21 %, in winter and summer milk samples, respectively, where AFM1 MRL is 0.5 µg/kg. The percentage of aflatoxin M1 samples exceeded the MRL of Egyptian regulation (ER) (2010) 2010/7136 last updated, are 78 and 100% in two examined seasons, where the limit for AFM1 MRL is 0.05µg/kg (Table 4).

Table 4: Positive aflatoxin M1 samples exceeding MRL according to different international regulations.

Sample category	Positive samples		Exceeding MRLs Eu		US FDA		Codex Alimentarius		ERS	
	No	%	0.05 µg/Kg		0.5 µg/Kg		0.05 µg/Kg		0.05 µg/Kg	
Winter	12	85	11	78 %	7	50 %	11	78 %	12	100 %
Summer	11	78	11	100 %	3	21 %	11	100 %	11	100 %

Codex Alimentarius (FDA/WHO) (2001), the US FDA, (2011) AFM1 ER: Egyptian regulation, 2010/7136*).*

DISCUSSION

Recently, there is much attention concerned on the control of AFM1 residues in milk, which is demonstrated by many factors. The importance of this topic is underscored by the various international organizations involved in monitoring AFM1 levels (Baskaya *et al.*, 2006).

In the present study, the examined level of aflatoxin M1 showed range of concentration between 0.0 - and 0.9 µg/kg with a mean value of 0.48± 0.31 µg/kg (480 ng/L) for winter milk samples. The examined level of aflatoxin M1 in summer milk samples showed range levels between 0.06 - 0.8µg/kg with a mean level of 0.23±0.30 µg/kg (230 ng/L). These data represented higher values in comparison with other study conducted on Egyptian raw milk samples by Mwanza *et al.* (2015), who detected aflatoxin M1 with a range of 8.52 to 78.06 ng/L, and also in South African milk samples, with a range of 5 to 120 ng/L. Our results are higher than those postulated by Amer and Ibrahim (2010) and Aiad and Aboelmakarem (2013) who demonstrated AFM1 in 38% and 40% of investigated raw milk with mean values of 49.7 ± 17.3 ng/l and 8.30–85.00 ng/kg, respectively, in Alexandria, Egypt. Similar findings was indicated by (Ghareeb *et al.*, 2013) who assayed AFM1 in 97.9% of examined raw milk samples with mean concentration of 62.9 ± 32.1 ng/l in Quena, Egypt. In previous study by Shaker and Elsharkawy (2014) on raw buffalo milk the mean concentration of AFM1 from Sohag was 64.49 ± 16.8 ng/L, with an average of 123.27 ng/L. In Assiut, the mean values of AFM1 in raw buffalo milk was 130.6 ± 29.9 ng/L, with an average of 250.79 ng/L. These data near to some extent to our results where the mean leveles of AFM1 in winter samples was 480 ng/L, and that in summer samples were 230 ng/L. In Aswan, Zakaria *et al.* (2019) documented shown results 49% positive for AFM1 from samples of raw milk and The positive milk samples have the values of AFM1 at range from 0.053 to 0.207 with mean ± SE of 0.1003 ± 0.008 ppb.

Moreover, the percentage were 85.5 and 78.5 % aflatoxin M1 in winter and summer milk samples, respectively. These results recorded higher frequency of occurrence than those obtained by Mwanza *et al.* (2015), with a detection incidence of 73.9% in Egyptian raw milk samples and 68% in South African raw milk samples. In a research was processed from September 2020 to March 2021, the samples were obtained from dairy shops and supermarkets at Cairo, Giza, and El-Minia governorates. AFM1 was detected in all examined raw milk with mean values of 40.27±3.996 ng/kg Esam *et al.* (2022). Their data lower than the obtained results in both values and for the frequency of distribution.

Data recorded in this work declared a higher incidence of AFM1 in winter milk samples (85.5%) than in summer milk samples (78.5%). Furthermore, the AFM1 concentration average was larger in winter milk samples (0.48± 0.31 µg/kg) than in Summer milk samples (0.23±0.30 µg/kg). In agreement with the obtained results Ismaiel

et al. (2020) reported that the AFM1 levels in samples obtained in winter were significantly ($P > 0.001$) which higher than those obtained in summer. These differences may be attributed to environmental and climatic changes, and also due to variation in the types and quality of buffaloes feed in different seasons. *Aspergillus flavus and Aspergillus parasiticus* fungi produced AFs as secondary metabolites when the temperatures ranged between 24 to 35°C and the moisture content over 7% Williams *et al.* (2004). The value and frequency of AFs are different due to the variable contamination sources of AFs in dairy products. Variable humidity and temperature situations from country to country or from various areas at the same country affect the origines of mycotoxin contamination Prandini *et al.* (2009). South Egypt has very hot summer and hot desert climate and very little precipitation rate. Due to high temperature (39–41°C) and humidity (58–61%), it can be expected that the amount of AFB1 is high in the animal feeds. Increasing daily intake of AFB1 polluted feed by dairy cattle will reflect with high incidence of AFM1 in milk Ghareeb *et al.* (2013). The milk samples investigated in the present work were collected from the Upper Egyptian city of Sohag, which is located in southern Egypt which had extremely high temperatures, extended dry seasons, and low availability of green fodder. Thus, farmers in these regions often depend on the use of feedstuffs that have been inappropriately stored, which leads to aflatoxin contamination. All these factors and, in particular, feeding animals AFB1-contaminated rations, led to the increased incidence of high AFM1 levels in milk in Upper Egypt. This is a serious considering a recent study that suggested that the most successful method to control AFM1 in the dairy products is to decrease AFB1 residues in the primary ingredients of feed produced for dairy animals.

Several nations have developed permissible limits for these metabolites in milk and dairy products that variable from one country to another and are affected by economic concerns. The European Commission regulation (165/2010) and the Egyptian standard set a limit of 50 ng/kg for AFM1 in row milk and liquid products. This limit is one order of magnitude lower than the 500 ng/kg limit set by the United States. In this study, among positive samples, 78% of the winter samples had AFM1 levels above the EU regulatory level (50 ng/L), whereas 100% of two examined seasons had concentrations above the early Egyptian Regulations level (1990) (0 ng/L) but 78% and 100% in winter and summer samples above late update of Egyptian standard, 2010/7136 (50 ng/L of AFM1). In comparison with Codex Alimentarius (FDA/WHO) (2001), 78% and 100% of the winter and summer samples had concentrations above the Codex Alimentarius regulatory limits. The percentage of aflatoxin M1 samples exceeded the MRL of the US FDA (2011) limit for AFM1 are 50 and 21 %, in winter and summer milk samples, respectively, where AFM1 MRL is 0.5 µg/kg. These data represented higher exceeded levels which recorded previously in the raw milk samples collected from Sohage and Assiut cities where the results declared that 93% of samples were over the permitted limit set by the European Commission (EC), whereas 3.3% of samples exceeded US FDA standard limit Shaker and Elsharkawy (2014). And also, higher than the samples were obtained from Cairo, Giza, and El-Minia dairy shops. AFM1 contaminated raw milk samples were 25.71%, which above the European and Egyptian regulation limits Esam *et al.* (2022). From this study, it is worthy to conclude that the variable levels of AFM1 in dairy products could be declared on the principals of climatic and seasonal variations and also due to change of geographical conditions. The malpractices of feed storage and farm management play a great role in such hazard Iqbal and Asi (2013). Moreover, the great difference of milk contamination with AFM1 was considered to be connected to variable issues including animal species, season, milking time, concentration of AFB1 intake, and amount of milk given by the

animal Assaf *et al.* (2019). Previously, the Ministry of Health legalized that the dairy products should be free from AFM1 (0 ng/ kg-1), in Egypt Iqbal *et al.* (2015). Recently, the Egyptian standard followed the EU regulation for liquid and row milk products Esam *et al.* (2022). Thus, strict legislations need to be applied by special authorizes in order to examine the presence of AFM1 in dairy products Ismaiel *et al.* (2020).

CONCLUSION

Our data combined with those from previous studies demonstrate the important aspects that must be considered with regard to AFM1 contamination levels in Egypt. Moreover, the findings of this work recommended that there is a serious public health hazard under Egyptian regulation due to AFM1-contaminated milk. All samples tested were positive for AFM1 and the concentrations were high compared with those obtained in studies previously conducted in Egypt. In addition, many samples were far above the legal limits set by other national organizations. Thus, the safety and quality of milk in Egypt, especially in Upper Egypt, must be strictly monitored to protect public health.

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بحث يرصد ويراقب الافلاتوكسين م١ في عينات الحليب الجاموسي الخام المباع في محافظة سوهاج في جمهورية مصر العربية في مختلف فصول السنة. تم جمع عينات الحليب من مارس الى اكتوبر ٢٠٢١. وتم فحصها من خلال. وقد تبين من خلال النتائج أن العينات تحتوي على معدلات عالية من السموم الفطرية تقدر بنسبة ٨٥,٥% في عينات الشتاء و ٧٨,٥% في عينات الصيف. وتتراوح معدلات العينات الايجابية مابين ٠,٠٦ الى ٠,٨ ميكروجرام \ كيلو جرام في عينات الشتاء و ٠,٠ الى ٠,٩ ميكروجرام \كيلوجرام في عينات الصيف. وقد لوحظ ان النسبة المئوية قد تخطت المواصفة القياسية المصرية المسموحة للافلاتوكسين ام١ في الحليب الخام للعام ٧١٣٦\٢٠١٠ وقد كانت النسب ك الاتي ٧٨% و ١٠٠% في الشتاء والصيف. وحسب النتائج للعينات في الفصلين يشكل ذلك خطر على صحة المستهلك والتي يتوجب من خلالها الفحص الدوري والدقيق والشامل لجميع منتجات الالبان لفحص السموم الفطرية الافلاتوكسين ام١ . ELISA