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Assessment of Aflatoxin M1 Contamination in Egyptian Milk and Cheese

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ABSTRACT

A screening survey of the presence of aflatoxin M1 (AFM1) was carried out on 170 samples of Egyptian dairy products. Selected samples included raw buffalo or cow milk, pasteurized milk, UHT milk, fermented milk, soft cheese, hard cheese, and processed cheese were analyzed by competitive ELISA technique. Out of 60 liquid milk and fermented milk samples analyzed, 12 samples (20%) were found to be contaminated with AFM1, at levels ranging from 10.12 to 86.54 ng/kg. Eight from the positive samples (66.6%) failed to reach the acceptable level of the European Commission Regulation (50 ng/kg). Buffalo's milk is safe regarding AFM1 contamination since all the buffalo milk samples were consistently negative. AFM1 was found in 22 samples (36.67%) of out 60 cheese samples at concentrations between 25.12 to 253.04 ng/kg. The highest mean value of AFM1, 231.39 ng/kg, was found in Ras cheese (hard type) samples followed by the processed cheeses with 114.76 ng/kg. Kariesh cheeses displayed the lowest levels, 53.32 ng/kg. Cheeses made from cow's milk showed higher AFM1 positive samples, while cheeses made from buffalo's milk tested were consistently negative. Regarding the cheese ripening age, out of 50 cow milk cheese samples, 26 were found to be positive to AFM1. The highest positive incidence was noted in long-term ripened hard cheeses (70%), while the lowest in fresh unripened cheeses (40%). It is highly recommended from the results that milk and cheese products have to be controlled periodically for AFM1 contamination.

Keywords: Aflatoxin M1; raw milk; UHT; cheese; buffalo; ELISA

INTRODUCTION

Aflatoxins are a group of toxic metabolite compounds produced by *Aspergillus flavus, A. parasiticus and A. nomius*. The contamination of milk and dairy products with these substances could be referred to the feeding of lactating animals with contaminated feedstuffs or directly as a result of intentional or accidental contamination with toxin producing molds during and after processing (Creppy, 2002 and Kokkonen, *et al*, 2005).

Among the aflatoxins groups, aflatoxin M1 (AFM1) is the hydroxylated metabolite derivative of aflatoxin B1 (AFB1), which is potentially toxic, and carcinogenic and it could be biotransformed by microsomial cytochrome P450, at the hepatic level, into the AFM1 and excreted later in this form in the milk, which could be consumed as drinking milk or processed to dairy products and harms the human health (Lopez *et al* 2001; Baskaya *et al* 2006 and Ardic *et al* 2009). The hazardous effect of aflatoxins on human health has been also reported by Bilandzic *et al* (2010), they reported that AFB1 is the most toxic aflatoxin and it is classified by International Agency for Research on Cancer (IARC), an organ of the World Health Organization (WHO) as group 1 of carcinogenic compounds (IARC,2002). Several studies have stated that the contamination level of milk and its products with AFM1 varied according to country, geographical area and season (Guo *et al* 2019). In Egypt, the use of green fodder rather than concentrated fodders in feeding animals results in a decreased level of AFM1 in the obtained milk (Aliabadi *et al*,2012). AFM1 in milk causes certain hygienic difficulties in processing of liquid milk and dairy products. Due to the fact that AFM1 is heterogeneously dispersed in milk and it is more linked to the protein fraction of milk (casein), separation of cream will ultimately increase the AFM1 concentration in dairy products by four folds (Prandini *et al* 2009; Picinin *et al* 2013 and Skrbic *et al* 2015).

Aflatoxins are heat resistant and can survive pasteurization, other heat treatments and mild acidic conditions applied during processing of cheese and fermented dairy products. The regulatory limits for the maximum level of aflatoxins in food varied among the different countries. However, Codex Alimentarius and European Commission issued that the maximum limit of AFM1 in milk and dairy products should not exceed 50 ng/kg (Codex Alimentarius Commission, 2001; European Commission Regulation, 2006). On other side, the US regulations put a level of maximum 500 ng/kg AFM1 in milk and dairy products (Omar, 2016). The regulations of the Egyptian Ministry of Health demand that liquid milk and dairy products should be free from AFM1 (Egyptian standards, 2003).

In a study carried out by Amer and Ibrahim (2010) on presence level of AFM1 in some cheese types produced in Egypt, it was found that soft and hard cheese samples showed AFM1 concentrations higher than that found in raw milk and processed cheese samples. The levels of AFM1 in the positive samples exceeded that of the Egyptian regulations. Other studies (Baskaya *et al* 2006 and Krstović *et al* 2018) showed the dependency of AFM1 concentration in tested cheese on type of cheese and applied production process and on moisture content in the final product. Application of contaminated milk powder in cheese making or contamination with fungi results in risk of aflatoxin

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synthesis from type B1, B2, G1 and G2 in cheese, although cheese itself has a low content of carbohydrates and cannot be considered as an optimum growth substrate for fungi (Barbiroli *et al* 2007).

Due to the rarity of the literature data on aflatoxin contamination of milk and dairy products in Egypt, the present work was designed to carry out screening for the contamination level of AFM1 in milk and cheese produced in Egypt applying ELISA technique (Enzyme linked immunosorbent assay) to contribute in measures of health risk assessment and for planning any necessary prevention actions.

MATERIALS AND METHODS

Materials

Sample Collection

Through 2019, a total of 170 samples of dairy products from different brands were randomly collected from food stores located in Cairo, Kalioubia, and Giza, governorates. Dairy products examined were liquid milk (Raw, pasteurized & UHT) and cheese (soft, hard and processed). Cheese samples tested were unripened (<1 month) and ripened (> 2months). The selected cheese samples included cow and buffalo cheeses. All samples were kept under refrigeration condition during the transport to laboratory.

Methods of Analysis

The samples were analyzed for the presence of AFM1 by ELISA method using RIDASCREEN Aflatoxin ELISA kits (R Biopharm AG, Darmstadt, Germany). Milk or cheese samples were prepared according to the method outlined in the ELISA kit.

Milk

Fat was skimmed from milk samples by centrifugation at 3500 rpm and 10°C for 10 min. A Pasteur pipette was used to remove the upper layer of cream. Skimmed milk samples were directly used in the test (100 µl per well). Limit of detection (LOD) for AFM1 in milk was <10 ng/l.

Cheese

Two grams of white or Ras cheese samples were homogenized and 40 ml of dichloromethane was added. The mixture was extracted by shaking for 15 min. The suspension was filtered and a 10 ml aliquot was evaporated under a nitrogen stream. The procedure of extraction was repeated using a mixture of 0.5 ml phosphate buffer saline (PBS), 0.5 ml methanol and 1 ml heptane. The obtained extract was subsequently centrifuged at 2500 rpm and 15°C for 15 min. The methanol layer was used for testing. LOD for AFM1 in cheese was <100 ng /kg.

ELISA was carried out on a Digital and Analog Systems microplate reader (Rome, Italy). Measurements were carried out photometrically at 450 nm, and the absorption was inversely proportional to the concentration of aflatoxins present in the samples.

RESULTS AND DISCUSSION

Raw milk and fermented milk

The concentrations of AFM1 in liquid and fermented milk samples are shown in Table (1). Out of 60 milk samples analyzed, 12 samples (20%) were found to be contaminated with AFM1, at levels ranging from 10.12 to 86.54 ng/kg. Eight from the positive samples (66.6%) failed to reach the acceptable level of the Codex Alimentarius Commission, (2001); European Community Regulation, (2006), defined as 50 ng/kg. As seen from the same table AFM1 levels in raw, pasteurized or UHT milk are similar, meaning that AFM1 is not destroyed during the pasteurization or UHT processes. The results of Tekinsen and Eken (2008) indicated that, 14 of 47 samples (29.8%) of commercial ultra-high temperature-treated milk were positive for AFM1. AFM1 levels in samples (31%) of UHT milk exceeded the maximum tolerable limit of the European Commission. Sumon *et al* (2021) reported that AFM1 was found in 78.6% of the tested milk and milk products and the recorded level ranged between 5.0 to 198.7 ng/L. Tested samples of pasteurized and UHT milk showed AFM1 levels in the range of 17.2 to 187.7 ng/L. and 12.2 to 146.9 ng/L, respectively.

Researches on the contamination level in milk produced in Jordan (Omar,2016) reported that the detected concentration level of AFM1 in cow milk samples was in the range of 9.71 to 288.68 ng/kg. AFM1 was detected at levels of 9.71-129.79, 14.60-216.78, and 18.0-288.68 for fresh cow milk, pasteurized cow milk, and full cream powdered milk samples, respectively. Survey studies on AFM1 contamination level of raw cow milk distributed in market of the Egyptian district Alexandria (Amer and Ibrahim, 2010) revealed that 38% of the tested samples showed a mean value of 49.7 ± 17.3 ng/l. The levels of AFM1 in 72 samples from raw milk and pasteurized milk in Iran have been reported, and 94.4% of tested milk samples were above the standard limits of Iran (100 ng/kg) and European Commission Regulation (50 ng/kg) (Rezaei *et al* 2015).

Aiad and Aboelmakarem (2013) reported that, AFM1 was detected in 26 % of tested yoghurt samples in the range of 11.40 - 98.80 ng/kg. Lower concentrations of AFM1 in yoghurt were reported by Sumon *et al* (2021) with a mean and range of 16.9 and 8.3-41.1 ng/l, respectively. Yoghurt samples showed lower AFM1 level than that found in liquid milk indicating the ability of some Lactic acid strains to reduce the level of AFM1 in yoghurt (El Khoury *et al*., 2011).

Table 1. Presence of Aflatoxin M1 (ng/l) in Liquid Milk and Fermented Milk Samples.

Milk type	Positive samples		Range		Mean
	N*	(%)	Min.	Max.	±SD
Raw buffaloes milk	0	0	0.00	0.00	0.00 ± 0.00
Raw cow milk	4	40	15.25	86.54	46.18 ± 12.14
Pasteurized cow milk	3	30	15.20	78.47	37.12 ± 8.60
UHT cow milk	3	30	10.12	70.68	33.29 ± 7.87
Yoghurt buffaloes milk	0	0	0.00	0.00	0.00 ± 0.00
Yoghurt cow milk	2	20	11.65	68.09	31.21 ± 13.77

***N, number of positive samples out of 10; AFM1, aflatoxin M1; SD, standard deviation.**

The results also showed that, buffalo's milk is safe concerning AFM1 contamination as all the samples were negative (Table 1). The obtained results agree with those reported by Santini *et al* (2013). Previous studies have pointed out to the ability of *Lactobacilli* strains to decontaminate milk from AFB1 and AFM1 by binding to these aflatoxins. *Lactobacilli* were more abundant in buffalo's compared to cow's milk (Oatley *et al* 2000; Pierides *et al* 2000 and Peltonen *et al* 2001). Another hypothesis that needs to be verified is, buffalo's animal has a better ability to detoxify bloodstream from aflatoxins. Another possible cause is, in Egypt majority of buffaloes are owned by small farmers, who feed their animals green fodder. Conversely, Guo, *et al* (2019) revealed that (62.5%) of the tested raw buffalo milk samples (85 samples) contained AFM1 in the range of 4 to 243 ng/kg and 8 samples of them (5.9%) showed values over the levels stated by the European Regulations. On other side, Hussein *et al*. (2008) found that the percentage of AFM1 contamination in buffalo milk was lower, than that found in cow's milk. The mean value of AFM1 was 44 ng /l in cow's milk compared with 27 ng /l in buffalo milk. Moreover, Rahimi *et al* (2010) reported that, 36% of raw cow milk, 8% raw buffalo milk samples were higher than the maximum tolerance limit accepted by European Committee/Codex Alimentarius Commission. Inconsistencies between the different studies may be explained by differences in fodder contamination levels among countries which can explain the geographical relations with AFM1 incidence in buffalo's milk. Hypothesizes that, variations in AFM1 incidence within one genetically stable animal (buffalo) might reflect changes in the exposure to AFB1 or to one or more protective factors. Geographical and temporal relations in AFM1 incidence have been observed in Egypt (Kamal *et al* 2019).

Cheese

Neuromeres factors contribute to contamination of cheese with AFM1. AFB1 contained in dairy cattle feed remains in cheese milk, growing of microorganisms such as *A. flavus* and *A. parasiticus* is conducive to synthesis of AF (B1, B2, G1 and G2), usage of contaminated dried milk with AMF1 for fortifying cheese milk during production, and finally, the attraction between AFM1 and casein micelles (Colak, 2007, Prandini *et al* 2009). In the same context, there is a global diversity concerning AMF1 specific limits in cheese. Switzerland, Austria, France, Iran (250 ng/kg), Netherlands (200 ng/kg), Italy (450 ng/kg) (Durakovic *et al* 2012; Cavallarin *et al* 2014; Fallah, 2010, and Skrbic *et al* 2015). Yet, some countries like Egypt and Romania have aimed to apply zero tolerance strategy to ensure full protection of the consumer (Anfossi *et al* 2011).

As shown in Table 2, the presence of AFM1 was detected in 22 (36.67 %) out of 60 cheese samples at concentrations between 25.12 to 253.04 ng/kg. The highest mean concentration of AFM1 (231.39 ng/kg,) was found in Ras cheese which is a hard type. Next were the processed cheeses with 114.76 ng/kg, and finally Kariesh cheeses, (soft cheese) which displayed the lowest level (53.32 ng/kg).

It noteworthy that, Ras cheese was four times higher than the accepted limit by European Committee. High contamination level may be attributed to heavy mold growth on the cheese surface during ripening. As expected, all buffalo milk samples were AFM1 negative, consequently all the samples of buffalo milk cheese either Kariesh or Domiati also were negative. Similar results were obtained by Montagna *et al* (2008).

Table 2. Presence of Aflatoxin M1 (ng/kg) in Different Cheese Types.

Cheese Type	Milk type	Positive samples N*	Positive samples (%)	Range Min.	Range Max.	Mean ±SD
Kariesh	Buffalo	0	0	0.00	0.00	0.00 ± 0.00
Kariesh	Cow	4	40	25.12	99.06	53.32 ± 11.81
Domiati	Buffalo	0	0	0.00	0.00	0.00 ± 0.00
Domiati	Cow	5	50	38.11	120.45	66.40 ± 20.15
Ras	Cow	7	70	85.08	253.04	231.39 ± 35.34
Processed	Mix	6	60	93.34	145.22	114.76 ± 14.52

***N, number of positive samples out of 10; AFM1, aflatoxin M1; SD, standard deviation.**

Long term ripened cheeses were the most frequently contaminated (70 %), while the lowest in fresh unripened cheeses ,40% (Table 3). These results agree with those reported by Montagna *et al* (2008). They stated that cheese samples ripened for medium and long term showed the most frequently contamination of the tested cheese samples (19.3% and 17.1%), respectively. On other side, the unripened cheese samples showed the lowest contamination level (13.5%). The vast range of AFM1 found in hard cheese can be attributed to several factors including: technique used for cheese manufacturing, the existence of some curd in whey that may influence concentration of AFM1, extraction technique applied and degree of milk contamination with AMF1 (Fallah *et al* 2009). The average concentration of AFM1 in soft, hard and processed cheese was 70.63, 132.24, and 52.52 as reported by Amer and Ibrahim (2010).

Table 3. AFM1 Concentration (ng/kg) in Cow Milk Cheese Samples at Different Ripening Age

Cheese type	Ripening age	Positive samples N*	Positive samples (%)	Range Min.	Range Max.	Mean ±SD
Kariesh	Fresh	4	40	51.05	67.13	54.10 ± 4.38
Domiati	Fresh	4	40	58.23	74.45	60.43 ± 4.57
Domiati	Long	6	60	52.19	118.48	71.55 ± 11.64
Ras	Short	5	50	22.47	90.54	67.48 ± 13.83
Ras	Long	7	70	85.43	260.76	242.82 ± 40.22

***N, number of positive samples out of 10; AFM1, aflatoxin M1; SD, standard deviation.**

Aiad and Aboelmakarem (2013**)** studied the existence of AFM1 in Kareish, Damietta, and Ras cheeses, found that 46, 53 and 56 % of the examined samples were positive with concentrations ranging from 6.20 - 70.26, 16.50 - 133.2 and 7.40 - 111.50 ng/kg, respectively. Similarly**,** Elkak *et al* (2012) detected AFM1 in 75 out of 111 cheese samples collected from the Lebanese market, with 13 of the positive samples were beyond the acceptable limit of 250 ng/kg. Following the same trend, 40 different types of cheese in Kuwait were analyzed for AFM1 contamination. 80 % of these samples contained AFM1 with concentrations ranging from 23.8 - 452 ng/kg, and only 2.5% of the positive samples showed higher levels of AFM1 than EU permissible limits (Dashti *et al* 2009). Also, Hassan *et al* (2018) reported that, 85 % of the cheese samples examined in Qatar were contaminated with AFM1, and the maximum content found was 217.1 ng/kg.

Occurrence of AFM1 in cheese samples has been found in samples obtained from South America and several European countries. In Serbia, for instance, 54 samples of hard and white type cheese were examined for AFM1 contamination and the results revealed that only 7 samples exhibited concentrations exceeded the acceptable limit (Skrbic *et al* 2015). Also, other studies in Turkey revealed that 38 % of the tested market cheese samples were contaminated with AFM1 at concentrations higher than the permissible EU limits (Yapar *et al* 2008). Moreover, samples of Brazilian Parmesan cheese were investigated for the presence of AFM1 and it was reported that the AFM1 toxin was detected in 18 out of evaluated 30 samples and the concentration in 8 of the positive samples exceeded the level of 250 ng/kg (Trombete *et al* 2014). Samples of Chinese buffalo milk have been found to be contaminated with levels of only 27.6 ng/kg, a much lower level than the allowed regulation limit in China (500 ng/kg) and could be considered as safe for consumers, Guo *et al* (2019). However, only 4 samples recorded values higher than 50 ng/kg, which exceeds the level set by the European Commission. In Spain, in a study carried out by Barrios *et al* (1996) on 35 samples of fresh, ripened and semi-ripened cheese, it was found that 16 samples (45.71%) showed AFM1 levels in the range of 20 to 200 ng/kg.

CONCLUSION

According to the results of this study, it can be considered that Egyptian milk and dairy products are highly contaminated with Aflatoxin M1 at levels exceeding regulatory limits. The hygienic quality of commercial milk products must be improved considerably, and this is especially true for cheese. Strict measures to prevent Aflatoxin M1 contamination starting from animal feed and during production and storage should be applied. In addition, it is important that feeds should be periodically tested for aflatoxins before feeding.

It deems useful to monitor the concentration of aflatoxins in dairy foods on a regular base by the designated governmental authorities and announce the results for public safety and awareness reasons. Meanwhile, educating food producers, processors and consumers with the hazardous effects of aflatoxins and the best way to minimize food contamination should be mandatory. Consumer rights organizations and consumers themselves should be vigilant and demand that producers, processors and government all work conscientiously to minimize the hazard of aflatoxin in animal and human foodstuffs.

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تقييم تلوث اللبن والجبن في السوق المصري بالأفلاتوكسين M1

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تم اجراء دراسة مسحية لوجود الافلاتوكسين M1 (AFM1) علي 170 عينة من اللبن ومنتجاته في السوق المصري. تضمنت العينات لبن بقري خام ، لبن جاموسي خام، لبن مبستر، لبن معقم بطريقة UHT، الالبان متخمرة، جبن طري، جبن جاف، جبن مطبوخ. تم تحليل العينات باستخدام تقنية ELISA. اشارت نتائج التحليل الي وجود 12 عينة (20 %) من بين 60 عينة من البان مختلفة ملوثة بـAFM1 بتركيزات تتراوح بين 10.12 الي 86.54 نانوجرام/كجم. ايضا وجد ان 8 عينات من العينات الايجابية (66.6 %) تجاوزت التركيز المسموح به من AFM1 وفقا لتشريعات الاتحاد الاوربي (50 نانوجرام/كجم). اشارت النتائج الي ان اللبن الجاموسي كان امنا فيما يتعلق بالتلوث بـ AFM1 حيث كانت نتائج تحليل جميع عينات اللبن الجاموسي سلبية باستمرار. أظهرت نتائج تحليل عينات الجبن الي وجود تلوث بـ AFM1 في 22 عينة (36.67%) من اجمالي 60 عينة بتركيزات تتراوح بين 25.12 الي 253.04 نانوجرام/كجم وبلغ اعلي متوسط لتركيز AFM1 قيمة 231.39 نانوجرام/كجم في عينات الجبن الراس (جبن جاف) يليه الجبن المطبوخ بتركيز 114.76 نانوجرام/كجم. اظهر تحليل الجبن القريش مستويات اقل من تركيز AFM1 حوالي 53.32 نانوجرام/كجم. أظهرت النتائج ان الجبن المصنعة من لبن بقري بها اعلي مستويات التلوث بالافلاتوكسين M1 ، بينما اشارت نتائج تحليل الجبن المصنعة من لبن جاموسي خلوها من AFM1. تم دراسة تأثيرعملية التسوية علي معدلات AFM1 حيث تم تجميع 50 عينة جبن مصنعة من لبن بقري ومختلفة في درجات التسوية، واوضحت النتائج ان 26 عينة اظهرت نتائج ايجابية في التلوث بالأفلاتوكسين M1 وكانت اعلي نسبة في الجبن المسواه لفترات طويلة (70%) بينما اقل نسبة تلوث كانت في الجبن الطازجة غير المسواه (40%) . من هنا يتضح اهمية اجراءات المراقبة علي عمليات انتاج اللبن والجبن بشكل دوري للتحكم في التلوث بالافلاتوكسين M1.