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Evaluation of Aflatoxin M1 in raw buffalo milk and some dairy products in Assiut governorate, Egypt

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ABSTRACT

Ninety random samples of raw buffalo milk, Kareish cheese, and yogurt (thirty of each) were collected from different localities in Assiut Governorate, Egypt. The samples were collected between July 2022 and April 2023 to analyze the presence of Aflatoxin M1 (AFM1) using an ELISA kit. The results revealed that out of the examined raw milk and dairy products (Kareish cheese and yogurt), 17 samples of raw milk (56.67%), 13 samples of Kareish cheese (43.33%), and 14 samples of yogurt (46.67%) tested positive for AFM1.The analytical results showed that AFM1 concentrations in the collected samples ranged from 4.35 to 55.48 ng/l, with an average count of 38.54 ± 3.045 ng/l for raw buffalo milk. For Kareish cheese, the concentrations ranged from 3.40 to 52.59 ng/Kg, with an average count of 44.12 ± 2.005 ng/Kg. In the case of yogurt, the concentrations ranged from 5.69 to 58.34 ng/Kg, with an average count of 32.86 ± 2.007 ng/Kg. The highest distribution frequency of AFM1 was observed at 10 ng/l (58.82%), 8 ng/Kg (61.54%), and 9 ng/Kg (64.28%) within the range of 10-50 ng/l or ng/Kg in the examined raw milk, Kareish cheese, and yogurt samples, respectively. It is worth noting that all examined raw milk, Kareish cheese, and yogurt samples exceeded the limits of Aflatoxin M1 established by Egyptian regulations for Mycotoxin in Foods, which require them to be free from AFM1. Additionally, there were three samples (17.65%) of raw milk, two samples (15.38%) of Kareish cheese, and two samples (14.29%) of yogurt that exceeded the European Commission Regulation (50 ng/Kg).

Keywords: Aflatoxin M1; Dairy products; Kareish cheese; Mycotoxin in Foods; Raw buffalo milk.

INTRODUCTION

Milk and dairy products are a highly essential food for human health at all stages of life, so efforts have been made to produce clean milk free from any harmful material as mycotoxins. Aflatoxins (AFs) are mycotoxin produced mainly by *Aspergillus* species as *A. flavus*, *A. parasiticus and A. nomius* (Creppy, 2002). The major classes of AFs were aflatoxin B1 (AFB1), aflatoxin B2 (AFB2) and aflatoxin (AFM1). AFM1 is a hydroxylated metabolite of aflatoxin B1 which has well established carcinogenic and mutagenic potentiality by many researchers Aflatoxin B1 was detected in milk and dairy products from dairy cattle feed on contaminated food with aflatoxin B1 which excreted in milk of lactating animals (Fallah, 2010). AFB1 is converted into AFM1 and excreted in milk, depending on many factors as milking process and the environmental conditions (Unusan, 2006). Kareish cheese is one of the most popular soft cheeses in Egypt due to its low price (Osman *et al*., 2012) Kareish cheese is manufacturing manufactured by farmers in small scale and under unhygienic measures in farmers houses that give a chance to produce that type of dairy products without examination of manufacturing milk and enough heat treatment. On the other hand AFM1may be concentrated in Kareish cheese during its storage Kareish cheese (Capei and Neri, 2007) that increase its risk to human health consumers. Prolonged storage time and bad storage conditions cause fungal growth, multiplication and secretion of mycotoxins (Stack and Carlson, 2003) depending on genetics of the animals. Aflatoxin M1 is thermostable and stable also in mild acidic conditions used in the production of cheese or other dairy products such as yoghurt has been accounted for the contamination of that's dairy products (Colak, 2007 and Oruc *et al.*, 2007). In Egypt and due to economic reason, there were a lot of animal feed stored in sea ports for several months exceed year that may be lead to fungal contamination due to humidity, high temperatures. This condition may favorable for yeast and mould growth that secrete of mycotoxins which cause human poisoning of mycotoxicosis. Aflatoxin B1 is carcinogenic, terato-genic and mutagenic (Iqbal *et al.*, 2010). Aflatoxin M1 which present in milk is considered harmful agent for human health because of its carcinogenicity and need for regular examination of milk and dairy products (Prado *et al.*, 2000; Iarc, 2002; Iqbal *et.al.*, 2014a , b).

The presence of AFM 1 in raw milk indicates that mycotic contamination of animals feed due to some environmental conditions as fluctuated temperature, high humidity. Prolonged storage time and bad storage conditions cause fungal growth, multiplication and secretion of mycotoxins (Stack and Carlson, 2003). The conversion of Aflatoxin B1 into metabolized AFM1, which can manifest in milk, is influenced by the genetic makeup of the animals (Unusan, 2006). Notably, Aflatoxin M1 is characterized as a thermostable toxin capable of with-standing heat

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treatments during food processing procedures (Park, 2002), thereby explaining its presence in various dairy products such as cheese and yogurt. Studies have shown that Aflatoxin M1 remains stable in white pickled cheese throughout storage and ripening processes (Oruc *et al*., 2007).

To mitigate the presence of AFM1 in milk, biological agents like *S. thermophiles* and *L. bulgaricus* have been proposed for reducing contamination levels. Consequently, fermented dairy products like yogurt are considered safer for consumption compared to heat-treated milk and certain dairy items like soft cheese (de Souza *et al.*, 2021; Murshed, 2020). Regular monitoring of milk and dairy products prior to human consumption is crucial to prevent or minimize AFM1 contamination (Esam *et al*., 2022). Therefore, this study aims to assess the prevalence of Aflatoxin M1 in raw buffalo milk, Kareish cheese, and yogurt in the Assiut governorate, Egypt. The levels of AFM1 detected will be compared against the maximum permissible limits set by Egyptian regulations and European standards, shedding light on the food safety implications associated with these dairy products.

MATERIALS AND METHODS

Collection of samples:

Ninety random samples of raw buffalo milk, Kareish cheese, and yoghurt (30 of each) were collected from different localities in Assiut governorate, Egypt from July, 2022 to April, 2023. Each sample was put in an ice tank with thermometer at maintain temp. at 4 °C. The samples were transferred directly to the laboratory for examination for AFM1.

Sample preparation for ELISA:

ELISA kit instructions were followed samples preparation for Aflatoxin M1detection the work was carried out.

Raw milk samples:

Twenty milliliters of raw buffalo milk sample were centrifuged at 3500 rpm/10 minutes. 100 µl of the milk were used directly after removal the fatty layer in the ELISA kit for AFM1 determination.

Cheese samples:

Two grams of a homogenized cheese samples were mixed with 40 ml of dichloromethane and then the whole mixture was shacked for 15 min. The suspensions was filtered and evaporated 10 ml from the filtrate at 60°C under N_2 stream, Then the formed preparation was filtered, and the suspensions (10 ml from the filtrate) was evaporated at 60°C under N_2 stream, then methanol, phosphate buffer saline (0.5 ml each) were added and 1 ml of heptane to re-dissolved the oily residue in a mixture. After that , the mixture was centrifuged at 2700 rpm/15 min. the upper layer of heptane was removed and 100 µl of the aliquot were diluted with 400 µl of kit buffer, put on the kit 100 µl of the diluted samples.

Yoghurt samples:

Ten grams of yoghourt sample were added mixed with100 ml of warm deionized water (20-25ºC) then centrifuged at 3500 rpm for10 min at 4ºC, then the upper creamy layers were removed and the lower phases were diluted with deionized water 20 times (v/v) then used for the quantitative assay.

Determination of Aflatoxin M1

The quantitative analysis procedure of AFM1 in milk and dairy products samples was performed as described by R-Biopharm GmbH (1999) manipulating a competitive ELISA (RIDASCREEN AFM1, R-Biopharm) based on antigen-antibody reaction of a competitive enzyme immunoassay. The strips were covered by specific antibodies to AFM1. (standard addition method was used to detect limit reach to 5 ng/kg that was made by adding 100 µl of sample solution and to 100 µl of standard 10 ng/kg to the wells to occupy the binding sites proportionately, then mixed gently and incubated at room temperature in the dark for 60 min. The liquid samples were poured out to the wells that filled with distilled water (250 µl). Then liquid was poured out finally and washed four times by washing buffer. After almost one hour, light absorption was read at 450 nm by ELIZA reader. Standard curve was used for AFM1 determination in milk and dairy products samples by competitive ELISA.

RESULTS

The study investigated a total of 90 samples, including raw buffalo milk, Kareish cheese, and yogurt. The results in Table (1) showed that, for raw buffalo milk samples, out of 30 samples tested, 17 were found to be positive for AFM1, accounting for 56.67%. The mean AFM1 concentration in positive samples was 38.54±3.045 ng/l, with a range from 4.35 to 55.48. The variation in AFM1 levels indicates a significant presence of the toxin in raw buffalo milk samples. Meanwhile, kareish cheese samples demonstrated that among the 30 samples analyzed, 13 tested positive for AFM1, which was representing 43.33% of the total. The mean concentration of AFM1 in positive samples was 44.12±2.005 ng/kg, ranging from 3.40 to 52.59. The results suggest a considerable occurrence of AFM1 in Kareish cheese, highlighting potential health risks associated with its consumption.

In the case of yogurt samples, 14 out of 30 were identified as positive for AFM1, making up 46.67% of the samples.The mean AFM1 concentration in positive yogurt samples was 32.86±2.007 ng/kg, with values ranging from 5.69 to 58.34. The findings indicate a notable presence of AFM1 in yogurt, emphasizing the importance of monitoring and regulating toxin levels in dairy products for consumer safety.

These results indicate that AFM1 contamination is present in all tested samples including raw buffalo milk, Kareish cheese, and yogurt samples collected from the market in different localities in Assiut governorate, Egypt. The concentrations of AFM1 varied within the examined samples, however all exceeded the established limits set by Egyptian regulations for Mycotoxin in Foods, which is free from any mycotoxin. The findings highlight the importance of monitoring and implementing measures to mitigate AFM1 contamination to ensure food safety and protect consumer health.

The occurrence of AFM1 (ng/l or ng/Kg) in the examined raw milk, kareish cheese, and yoghurt samples is presented in Table (2). The results provide an overview of AFM1 concentration ranges (ng/L or ng/kg) and the percentage of positive samples recorded in different dairy products (raw buffalo milk, Kareish cheese, and yogurt). The data is categorized based on the concentration range of AFM1 and the percentage of positive samples recorded for each sample type of the three dairy products. It gives a clear comparison of AFM1 contamination levels across the different products.

For raw buffalo milk, among the positive samples, 23.53% had AFM1 concentrations above 10 ng/L or ng/kg, while 58.82% fell within the range of 10-50 ng/L or ng/kg. Only 17.65% of samples had AFM1 levels below 50 ng/L or ng/kg. In the case of kareish cheese, similar trends were observed in which 23.08% of samples were exceeding 10 ng/L or ng/kg, 61.54% falling within the 10-50 ng/L or ng/kg range, and 15.38% below 50 ng/L or ng/kg. Meanwhile, the Yoghurt samples recorded AFM1 concentrations greater than 10 ng/l, where 21.43% (3 samples) had 64.28% (9 samples) had AFM1 concentrations ranging from 10 ng/l to 50 ng/l. 14.29% (2 samples) had AFM1 concentrations less than 50 ng/l. In total, 17 raw buffalo milk samples, 13 kareish cheese samples, and 14 yoghurt samples were positive for AFM1, accounting for 100% in each category.

These findings provide insights into the occurrence patterns of AFM1 concentrations in the examined samples, highlighting the prevalence of AFM1 contamination at varying levels in raw milk, Kareish cheese, and Yoghurt.

Compliance with AFM1 Regulations in Positive Dairy Samples

Data on investigated positive sample types are displayed in the table (3), with particular attention paid to the proportion of samples that meet Egyptian requirements and are free of AFM1 contamination, as well as samples that exceed the >50 ng/kg European Commission (EC) regulation limit. In Raw Buffalo Milk, all 17 positive raw buffalo milk samples (100%) exceeded Egyptian regulations for AFM1 contamination. Additionally, 3 samples (17.65%) surpassed the EC regulation limit of >50 ng/kg.

Kareish cheese, similarly, all the thirteen positive Kareish cheese samples (100%) were found to exceed Egyptian regulations for AFM1. Out of these, two samples (15.38%) exceeded the EC regulation threshold of >50 ng/kg. However, in the case of yogurt, all the 14 positive samples (100%) surpassed the Egyptian regulations (1990) for AFM1. Among these, two samples (14.29%) exceeded the EC regulation limit of >50 ng/kg.

Table (1): AFM1 Concentrations in raw milk, kareish cheese, and yoghurt samples collected from different localities in Assiut governorate, Egypt.

Investigated Sample types	Samples No	AFM1-Positive samples		AFM1Concentration (ng/l or ng/Kg)		
		No	%	Mean values†	Minimum	Maximum
Raw buffalo milk	30	17	56.67	38.54 ± 3.045^{b}	4.35	55.48
Kareish cheese	30	13	43.33	44.12 ± 2.005^{a}	3.40	52.59
Yoghurt	30	14	46.67	32.86 ± 2.007^{b}	5.69	58.34

†All data are in Mean ±SD; means with the different superscript letter are significant different at level $p \leq 0.05$

Table (2)*:* Occurrence of AFM1 (ng/L or ng/Kg) in the examined samples.

AFM1 conc range (ng/L or ng/Kg)	Positive sample recorded					
	Raw buffalo milk		Kareish cheese		Yoghurt	
	No.†	%	No.	%	No.	%
>10	4	23.53	3	23.08	3	21.43
10-50	10	58.82	8	61.54	9	64.28
< 50	3	17.65	2	15.38	9	14.29
Total	17	100	13	100	14	100

†No., number of positive sample.

Table 3: Aflatoxin M1 (AFM1) levels (ng/l or ng/Kg) in examined samples that exceed Egyptian permissible limits and EC/Codex.

Investigated positive sample types	Exceeding Egyptian regulation free from AFM1		Exceeding EC regulation (>50 ng/ Kg)	
	No.[†]	%	No.	%
Raw buffalo milk	17	100	3	17.65
Kareish cheese	13	100	2	15.38
Yoghurt	14	100	2	14.29

[†]No., number of positive sample.

The widespread occurrence of Aflatoxin M1 (AFM1) contamination in the dairy products under investigation is emphasized by these results. Conseq-uently, to guarantee adherence to legal requirements and protect consumer health, stronger monitoring and control procedures are needed.

DISCUSSION

Aflatoxins are toxic metabolites produced by *Aspergillus* species such as *A. flavus* and *A. parasiticus*. They contaminate animal feeds such as corn, cottonseed, peanut products, and occasionally soybeans or distiller's grains. Cows that consume feed containing aflatoxin B1 may produce milk with elevated levels of aflatoxin M1 raising concerns about the safety of dairy products for human consumption. These toxins exist in dairy products, pose a serious threat to food safety and animal health, particularly when present in animal feeds such as corn, cottonseed, and peanut products.

The Positive samples contaminating with AFM1 of the examined raw milk, Kareish cheese and Yoghurt samples for AFM1 were 17 (56.67%), 13 (43.33%) and 14 (46.67%) respectively (Table 1). While statistical analytical results of AFM1(ng/l or Kg) concentrations ranged from 4.35, 3.40 and 5.69 to 55.48 , 52.59 and 58.34 with an average count of 38.54±3.045 , 44 .12±2.005 and 32.86±2.007 for the examined samples respectively (Table 1). Results in Tables (2) showed that the highest distribution frequency of AFM1 were 10 (58.82 %), 8 (61.54 %) and 9 (64.28%) that lied within the range of 10-50 ng/l or Kg in the examined samples respectively.

The presence of AFM 1 in raw milk indicated that mycotic contamination of animals feed due to some environmental conditions as fluctuated temperature, high humidity playing an important role in fungal growing and toxins AFs the synthesis of AFs (Stack and Carlson, 2003) Animal genetics is an important factors of the animal Aflatoxins B1 respond and its conversion and converted into metabolized AFM1 that appearing in milk, (Unusan, 2006). Aflatoxin M1 is thermostable which can resistant heat treatments (Park, 2002) that explain the appearance of AFM1 in dairy products as cheese and yogurt.

Aflatoxin M1 demonstrated remarkable stability in white pickled cheese for a period exceeding 90 days, maintaining its integrity throughout the storage and ripening phases. Oruc *et al*. (2007) highlighted the efficacy of Lactic acid bacteria, specifically *Lactobacillus* spp., in the decontamination of AFM1 from milk through detoxification processes. This declaration is supported by research conducted by El Khoury (2011), El-Nezami *et al*. (1998), Govaris *et al*. (2002), Abolfazl (2005) and Tooraj *et al*. (2023), all of whom attributed the low levels of AFM1 found in yogurt to the presence of lactic acid bacteria, particularly *Lactobacillus* spp., employed in the yogurt manufacturing process.

The results presented in Table (3) indicate that the levels of AFM1 (ng/L or ng/kg) in all examined raw milk, Kareish cheese, and yogurt samples exceeded the permissible limits set by Egyptian regulations (1990), which mandate that they must be free from AFM1. Specifically, 3 samples (17.65%), 2 samples (15.38%), and 2 samples (14.29%) were found to exceed the EC regulations (>50 ng/kg). Similar findings were reported by Balata and Bahout (1996), Elgerbi *et al*. (2004), Oveisi *et al*. (2007) and Aiad and Abo El-Makarem (2013). However, higher levels of contamination were reported by Motawee *et al*. (2004a and b).

CONCLUSION

Aflatoxin M1 (AFM1) is a metabolite produced by Aspergillus species, commonly found in feed ingredients like corn, cottonseed, soybeans, distiller's grains, and peanut products. When lactating cows ingest feed contaminated with aflatoxins, these toxins are excreted in their milk, leading to the presence of aflatoxins in dairy products such as cheese and yogurt produced using contaminated milk. These toxins pose significant risks to food safety and human health. Given their dangerous consequences and the well-documented carcinogenic effects of mycotoxins on human health, it is crucial to regulate and monitor their presence in the food supply chain. To effectively address this issue, several key points need to be considered: ensure feeds are free from contamination by yeast and molds through the implementation of good manufacturing and storage practices; employ chemical, physical, and biological treatments to reduce aflatoxin M11 concentrations in feed; prevent fluctuations in temperature, high humidity, and moisture in animal feed storage areas; conduct periodic examinations of dairy animal feeds to monitor AFM1 levels; regularly test raw milk for the presence of AFM1 contamination; implement Hazard Analysis and Critical

Control Points (HACCP) systems in dairy farms and production facilities to prevent toxin production throughout processing, packaging, and storage; store dairy products away from temperature and humidity fluctuations, especially during the summer season, to maintain product integrity and conduct regular insp-ections of imported dairy products before allowing them into the country. By adhering to these recommendations and maintaining strict quality control measures at every stage of the dairy production process, the risk of aflatoxin contamination in dairy products can be minimized, thus safeguarding consumer health and well-being.

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تقييم الأفلاتوكسين M1 في حليب الجاموس الخام وبعض منتجات الألبان في محافظة أسيوط، مصر

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تناولت الدراسة تقدير معدل ونسبة تواجد الأفلاتوكسين ام بتسعون عينة عشوائية من اللبن الخام والجبن القريش واللبن الزبادى بواقع (30 عينة لكل منها) من أماكن مختلفة فى محافظة أسيوط ، مصر.فى الفترة من يوليو 2022 إلى أبريل 2023 تم تحضيروتجهيز جميع العينات بواسطة ELISA kit.لتقديرمعدل ونسبة تواجد الأفلاتوكسين م 1. وأظهرت النتائج ان العينات التى كانت تحتوى على الأفلاتوكسين م1فى اللبن الخام والجبن القريش واللبن الزبادى كان عددها ونسبتها 17(56.67 %) و13 (43.33%) و 14 (46.67%)على التوالى. وكان معدل تواجد الأفلاتوكسين م1 بال (نانو جرام / لتر أو كجم) فى اللبن الخام والجبن القريش واللبن الزبادى يتراوح من 4.35 إلى 55.48 بمتوسط عدد3.045± 38.54 و 3.40 إلى 52.59 بمتوسط عدد2.005± 44 .12 و 5.69 إلى 58.34 بمتوسط الع2.007 ± 32.86 على التوالي. و كان أعلى معدل توزيع تكراري للأفلاتوكسين م11فى اللبن الخام والجبن القريش واللبن الزبادى هو 10(58.82 %) و8(61.54%) و9(64.28%) على التوالي. وكانت جميع عينات اللبن الخام والجبن القريش والزبادي التي تم فحصها غير مطابقة المواصفات القياسية المصرية والتي تنص على أنه يجب أن يكون اللبن الخام ومنتجات الألبان المختلفة خالية تماما من بقايا الأفلاتوكسين. بينما كانت هناك فى اللبن الخام والجبن القريش واللبن الزبادى 3 (17.65٪) و 2 (15.38٪) و 2 (14.29٪) أعلى من النسب المسموح بها بالمواصفات القياسية للاتحاد الأوربي والتي تنص على أنه يجب ألا تزيد نسبة الأفلاتوكسين فى كلا من اللبن الخام ومنتجات الألبان عن (50 نانوجرام / كجم) . و تمت مناقشة الأهمية الصحية لتواجد بقايا الأفلاتوكسين فى الألبان ومنتجاتها والطرق المختلفة لمنع تلوث الألبان ومنتجاتها ببقايا الأفلاتوكسين م1.