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Research Article
Occurrence of Aflatoxin M_1 in Cheese and Yoghurt Marketed at El-Fayoum Province, Egypt

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Abstract
Background and Objective: Nowadays, Aflatoxin M_1 is the most significant mycotoxins globally established on the prevalence, especially in milk and dairy products and destructive impacts on people's health and dairy industry business. For these causes, an inspection survey of aflatoxin M_1 (AFM_1) was accomplished on one hundred skim milk cheese (kareish), fifty flavoured drinking yoghurt and fifty flavoured cheese samples. **Materials and Methods:** The quantitative analysis of AFM_1 was achieved using solid-phase column extraction C_{18} for clean-up followed by the ELISA method. **Results:** Eighty percent of the examined skim milk cheese was positive, while, it could not be found in all the examined flavoured drinking yoghurt and flavoured cheese (<50 ng kg^{-1}). In the skim milk cheese, AFM_1 levels were ranged from 59.1-875.4 ng kg^{-1}. **Conclusion:** The low AFM_1 level in analyzed drinking yoghurt and flavoured cheese samples indicated that they apply high-quality raw milk to process drinking yoghurt and using non-dairy fat ingredients in flavoured cheese processing. This study demonstrated that the data of the 1st survey on the existence of AFM_1 in flavoured drinking yoghurt and flavoured cheese consumed in Egypt.

Key words: Aflatoxin M_1, kareish cheese, drinking yoghurt, flavoured cheese, solid-phase extraction, ELISA

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The ultimate communal kind of skim milk fresh soft cheese in Egyptian countries is kareish cheese. The elevating need for it by Egyptian consumers is specific to cheap and its high protein content. It is made from raw fat-free buffaloes or cow's milk[1,2]. Drinking yoghurt with various flavours is scrumptious yoghurt newly, dominant in Egyptian shops and consumed by many people. Also, it is a healthful drink, comprising nearly all the milk supplements present and great therapeutic characteristics[3,4].

Mycotoxins are subaltern products of mycotoxigenic moulds that are released under adverse environmental factors like high temperature, humidity and high moisture percent during the multiplication of these fungi. The most predominant mycotoxigenic fungi include some *Aspergillus* members, *Fusarium* spp. and *Penicillium* spp., which produced toxins as (Aflatoxins, Ochratoxin A and Citrinin) under bad climatological states of storage in agronomical products and food[5].

Aflatoxins are the highest risk mycotoxins, which have carcinogenic, immunosuppression, genotoxic and teratogenicity effects. They are produced by aflatoxigenic *Aspergillus (flavus*, *parasiticus* and *nomius*) in different food. There are two types of aflatoxins (B, G), AFB, which is produced by *A. flavus* and AFG and B produced by *Aspergillus, parasiticus* and *nomius*[6-8]. The worldwide type of health hazard for humans and animals is AFB_1 and the repeated exposure to these toxins resulted in the potentials of cancers, particularly liver[9]. Besides, Claeys *et al.*[10] and Iqbal *et al.*[11] classified AFB_1 as group one human carcinogen and mainly detected in food, feeds, meat and converted to AFM_1 in milk.

Moreover, the feeding of animals contaminated feeds with AFB_1, which can be metabolized by enzyme Cytochrome P (CYP450) in hepatic tissue, converted to AFM_1 and excreted into the animal milk. There is a direct association between the concentration of milk AFM_1 and eating feed containing AFB_1[12,13]. It has been determined that about 3.25% (average) of AFB_1 found in animal ration convert to AFM_1 in milk. The cell toxicity, gene toxicity and cancer-causing effects are clearly explained for AFM_1[6].

In milk products, the existence of AFM_1 is an international concern since these products are regularly used by people and so could be important vehicles for inserting the Aflatoxins into human food[14]. Consequently, several countries have harmonized the maximum allowable concentration of AFM_1 in milk and its derivatives to keep consumers safe, particularly children. These regulations vary from one nation to another by the fact of profit view. The Commission of Europe has agreed to a limit (50 ng L^{-1}) for aflatoxin M_1 in milk[15]. Based on the Institute of Standards and Industrial Research of Iran, Kamkar *et al.*[16] reported the maximum limit for aflatoxin M_1 10-times (500 ng L^{-1}) greater than the European Commission (EC) limit.

Cheese is susceptible to the multiplication of moulds and produces mycotoxins[17,18]. Aflatoxins in cheese might be due to these causes: milk containing residue of aflatoxin M_1 from which cheese has made. Growing the fungi like *Aspergillus* spp. on cheese and secrete aflatoxins (B_{1-2}-G_{1-2}). Existence of aflatoxin M_1 in dried milk used in cheese manufacture[19,20]. Aflatoxin M_1 is not influenced significantly by heat treatment as, sterilization and pasteurization[21,22]. Several researchers concluded that AFM_1 remains stable in stores of different cheese types[23].

The wide use of analytical methods applied for the detection of aflatoxin M_1 is ELISA, Thin Layer Chromatography (TLC), Immune-affinity Column-fluorometric and High-Performance Liquid Chromatography (HPLC). ELISA achieved regular screening for large scale for the following reasons, fast, simple, easy application and the cheapest prices[24]. Colak *et al.*[25] concluded that competitive ELISA is an accurate manner for analysis of AFM_1 in cheese. Furthermore, the exposure risk of humans to AFM_1 in milk and milk products may predispose the risk factor to hepatic cancer for consumers. Hence, the estimation of Daily Intake (EDI) of AFM_1 and its Hazard Index (HI) was calculated. If the HI value was <1, it means that AFM_1 intake from the examined samples did have a risk for these product consumers[26,27].

In a growing world include Egypt, there is scarce knowledge and little data have been published on the prevalence of aflatoxin M_1 in skim milk cheese, drinking yoghurt and flavoured cheese. So, this research aimed to determine aflatoxin M_1 concentration in skim milk soft cheese and drinking yoghurt and flavoured cheese (for the 1st time) offered in the Al-Fayoum, Egypt, a comparative study of obtained results with the international legal limit for AFM_1 authorized by EC. All these data help in the detection of potential risks, posed to human health resulting from the utilization of these products that are contaminated with AFM_1.

MATERIALS AND METHODS

Materials: This study was carried out from June, 2020 to December, 2020. One hundred skim milk soft white (kareish) cheese, fifty flavoured drinking yoghurt (25 strawberry and banana flavours for each) and fifty (pepper and Tomato) flavoured cheese samples of plant origin were randomly acquired from various retail outlets and markets in AL-Fayoum province, Egypt. The quantity of kareish and flavoured cheese samples was 250 g for each, while in drinking yoghurt samples, each bottle about (440 mL) in an excellent state by observation. The obtained samples were transferred to the lab in a careful proper condition.

Methods: The measurable examination of aflatoxin M_1 was based on Competitive Enzyme Immunoassay by RIDASCREEN® AFM_1 30/15 (Art. no: R1111, R-Bio-Pharm, Darmstadt, Germany) kit. The utilized chemicals were obtained by the kit industrialist. Chloroform, methanol, dichloromethane and C_7H_{16} were provided from Merck. For achieving recovery study, AFM_1 standard was got from Sigma Company (Sigma-Aldrich, A6428). This standard was obtained from *Aspergillus flavus*. AFM_1 stock solution (50 mg mL^{-1}) was done in a methanol/chloroform mix (81:19 v/v) and kept at -20°C. Before use, it was diluted with methanol/chloroform (1:1 v/v) at required concentrations according to Lopez *et al.*[19].

Extraction and clean up using solid-phase extraction: The AFM_1 in the investigated samples were extracted with 75 mL chloroform, 1 mL saturated NaCl solution and 5 g Celite Hyflo Supercel for 45 min with unceasing shaking. The mixture was filtered using paper 110 mm (Schleicher and Schuell, Germany). The extract of chloroform was put into a 250 mL flask, evaporated to dryness in H_2O bath (Edelstahl, UK) at 30°C. One mL methanol, 30 mL distilled H_2O and 50 mL hexane was supplemented to the residue[28].

By separating the funnel, the mixture was relocated, then washed two times with 10 mL distilled H_2O and shaken for fifteen seconds. The lower layer was collected and utilized in the cartridge C_{18} (Machery and Nagel, Germany) for clean-up. The cartridge C_{18} was primed by adding 10 mL CH OH followed by 10 mL dist. H_2O. The collected layer gently pulled through the cartridge. Ten mL of distilled H_2O followed by 10 mL of C_6H_{14} were added as washing solution and pulled through the column. Three mL of eluting dichloromethane/acetone (3:1) was pulled through the cartridge. Finally, elute was collected in a 4 mL glass vial tube and evaporated till dry.

Determination of AFM_1 using ELISA: Standard solutions (100 µL) and extracted samples were poured into independent microtiter wells to seal the binding places and kept in the absence of light at (25°C) for 60 min. Then, the solution was squandered and wells were washed by Buffer of washing (250 µL) twice. In the following, 100 µL of the watered enzyme conjugate was supplemented to fill free binding places and kept in the absence of light at room temperature for 15 min. Again, the wells were washed twice to remove any free enzyme conjugate. Subsequently, 100 µL of substrate/chromogenic was added and put in the absence of light at 25°C for 15 min. Bound enzyme conjugate converted the colourless chromogen to blue output. Finally, 100 µL of the stop solution (1N H_2SO_4) was supplemented into the wells and the colour became yellow. The absorbance was determined at λ equal 450 nm in the plate reader of ELISA (ELX800, Bio-Tek Instruments, USA) versus air blank within 15 min. According to the RIDASCREEN kit directions, the lower detection limit is (50 ng kg^{-1})[29].

Recovery study: For the validation of the method, a recovery study was achieved by spiking known concentrations (50, 150, 250 and 450 ng kg^{-1}) of AFM_1 into extracted samples just before the examination. The sample preparation and ELISA test steps were done as described above. All experiments were achieved using four samples/each treatment. Under these conditions, the mean recovery scores in spiked samples were 99.00%, with a coefficient of variation of 8.5%. Based on the kit directions, the recovery rate in the examined samples is almost 100.00%, with a mean coefficient of variation of 1.1%.

Estimated daily intake (EDI) calculation: The estimation of dietary exposure to AFM_1 was calculated from the mean concentration of toxin in skim milk cheese (ng kg^{-1}). The daily intake of this cheese is 22 g and the mean adult person's bodyweight is 60 kg[26,30]. Estimated AFM_1 Daily Intake was achieved by these Eq.:

$$\frac{\text{EDI (ng kg}^{-1})}{\text{body weight (day)}} = \frac{\text{AFM1 mean (ng kg}^{-1}) \times \text{Amount of consumed kareish cheese}}{\text{Mean adult person body weight}}$$

$$\text{HI} = \frac{\text{EDI (ng kg}^{-1}\text{ b.wt./day)}}{\text{0.2 reference dose (ng kg}^{-1}\text{ b.wt./day)}}$$

Statistical analysis: The p-value for significant difference between the mean values of AFM_1 concentration was predestined by T-test through Excel of Microsoft 365 enterprise. The p (less than 0.05) was approved as a significant value and 95.00% Confidence Interval.

RESULTS AND DISCUSSION

The AFM_1 prevalence in the investigated kareish cheese depicted in Table 1 and 2. Aflatoxin M_1 was found in 80.00% of the examined skim milk cheese, ranging between 59.1-875.4 ng kg^{-1} and AFM_1 was not detected (<50 ng kg^{-1}) in all examined drinking yoghurt and flavoured cheese samples.

Table 1: Existence of AFM_1 in the examined samples and its agreement with EC regulation (n = 200)

	Positive samples	Concentration (ng kg^{-1})		Exceed (EC) regulation (>50 ng kg^{-1})
Sample type	No. (%)	Range[a]	Mean±SEM	No. (%)
Kareish cheese	80 (80.00)	59.1-875.4	247.7[a]±22.1	80 (80.00)
Flavored drinking yoghurt	0 (0.00)	<50[b]		0 (0.00)
Flavored cheese	0 (0.00)	<50[b]		0 (0.00)

n: Number of the examined samples, a: Minimum-maximum values, SEM: Standard error of mean and No.: Number of positive samples. The different superscript letters in the same column indicate the significant difference (p<0.05)

Table 2: Concentrations of aflatoxin M_1 in the examined samples

	Range of aflatoxin M_1 (ng kg^{-1})					
	<50[a]	50-150	151-250	251-450	451-650	>650
Sample type	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Kareish cheese	20 (20.00)	14 (14.00)	37 (37.00)	10 (10.00)	8 (8.00)	11(11.00)
Flavoured drinking yoghurt	50 (100.00)	0 (0.00)				
Flavored cheese	50 (100.00)	0 (0.00)				

No.: Number of positive and negative samples and a: Negative samples

Aflatoxin M_1 concentration in 80.00% of the examined kareish cheese was exceeded the critical value (50 ng kg^{-1}) which, was legislated by EC[6].

There was a significant difference (p<0.05) between the mean value of AFM_1 concentration of kareish cheese and flavoured drinking yoghurt samples, also between the mean value of AFM_1 concentration of kareish cheese and flavoured cheese. As reported in Table 2, there is a considerable variation in the AFM_1 concentration. Most of the examined kareish cheese samples (51.00%) contained AFM_1 at 50-250 ng kg^{-1}, while the 10, 8.00 and 11.00% of the examined kareish cheese samples were contaminated with AFM_1 at concentrations, 251-450, 451-650 and >650 ng kg^{-1}, respectively. In addition, AFM_1 was not detected (<50 ng kg^{-1}) in 20.00% of the examined kareish cheese samples. The Estimated Daily Intake (EDI) and Hazard Index (HI) for AFM_1 from the eating of Egyptian kareish cheese were 0.091 and 0.455 ng kg^{-1}, respectively.

Taken into account, the preferred compatibility of AFM_1 for milk casein portion, a high toxin amount, may happen in curd processing[17]. Investigations indicated that the concentration of AFM_1 is four times greater in cheese than in milk, which is utilized in its production[22]. So, cheese could be the highest origin of aflatoxins among milk products. An elevated AFM_1 mean value in the examined skim milk cheese was recognized since multiple surveys recorded the great AFM_1 existence in plant-made and farmers-made cheese from many areas[18,26,31].

Current results agree with those recorded in many countries. In Libya, Elgerbi *et al.*[32] found that the AFM_1 in 75.00% of twenty cheese samples, with range 0.11 to 0.52 µg kg^{-1}, while the percentage of AFM_1 in the examined 20 skim milk cheese samples was 55.00%[1]. In Brazil, AFM_1 was found in 74.70% of white soft cheese samples collected from Minas Gerais, the range from 0.02-6.92 µg kg Minas Gerais and 26.70% samples were greater than 0.25 µg kg^{-1} [33]. In another study, the AFM_1 was found in (80.00%) of the investigated samples in Kuwait[31]. Hosny *et al.*[34] found that 33.3% of the examined skim milk cheese AFM_1 polluted with an average of 0.027±0.009, a minimum of 0.01 and a maximum of 0.04. The forgoing results indicated that the dairy milk used for processing skim milk cheese must be free from AFM_1 to avoid pollution of cheese and safe human health. The existence of AFM_1 in animal milk mainly resulted from the consumption of contaminated feed with AFB_1.

In the present study, the findings were compared with the international limit of EC authorities. In this respect, Kamkar[35] detected AFM_1 in 82.50% of the examined skim milk cheese samples with average levels of 0.41 µg kg^{-1} and (60.5%) of these samples were incriminated AFM_1 greater than the upper allowable international limit (0.25 µg kg^{-1}). While Rahimi *et al.*[36] recorded that 53.4% of the examined cheese samples contaminated with AFM_1 at the range from 82-1254 ng kg^{-1} and 31.80% of these samples were overrun the limit 250 ng kg^{-1}.

Current findings were higher than obtained by Montagna *et al.* [37], who found AFM_1 in 16.6% of soft cheese samples with an average of 88.6 ng kg^{-1}. In another study, 15% of the investigated soft cheese samples were found positive for AFM_1 and its concentration was (>50 ng kg^{-1}) in all samples[38].

Moreover, the AFM_1 was found in 39 soft cheese sample[21]. They resulted that 11 (28.2%) samples were polluted with AFM_1, with a maximum concentration of 188.4 ng kg^{-1}. Whereas, Atanda *et al.*[39] revealed that no detectable concentrations of AFM_1 were recorded in soft cheese. Similarly, Martins *et al*.[40] not detected AFM_1 in soft cheese and dried milk using the HPLC method. Discipline programs for operators and the exactness of (HACCP) system are necessitous for satisfying the safe food articles[41-47].

Vaz *et al*.[48] reported that the hazardous compounds become concentrated in food through processing. The Enriched Factor (EF) of a product is the significant key factor for determining exposure. Although the AFM_1 levels in kareish cheese have been reported to be higher than European legislation, many of the experiments concerning EFs have been performed using artificially contaminated milk. The EFs differed based on the cheese kind plus the source of milk contamination. The enriched factor was ranged from 4.1-4.9. Therefore, cheese using contaminated milk with AFM_1 was just about three folds greater than the manufactured fresh milk[18,49].

The variations in detected AFM_1 concentrations in forgoing studies may be related to different factors included cheese preparation, ripening, changes in season's climates, storage and packing[29]. It has been reported that the higher levels of milk AFM_1 processed during cold times[50].

Hassan and Kassaify[51] recorded a higher EDI of 0.14 ng kg^{-1} daily body weight in the examined cheese samples. When the HI value was <1, it means that the risk degree for hepatic cancer in Egyptian kareish cheese consumers is not common. HI obtained in this investigation was greater than the number recorded by Shahbazi *et al*.[27], who reported that the EDI is affected by weather and the technique of AFM_1 determination.

The low aflatoxin M_1 level (<50 ng kg^{-1}) in flavoured cheese of plant origin samples may be attributed to mixing ingredients (non-dairy fats and/or proteins) for cheese processing to conform to particular specification with low cost[52]. Regarding drinking yoghurt samples, Atasever *et al*.[53] detected that the AFM_1 levels were greater than the international limit of 50 ng kg^{-1} in 13.6% of the examined drinking yoghurt samples. They revealed t hat the min of AFM_1 was 6 ng kg^{-1}, the max was 264 ng kg^{-1} and the mean value 36.5 ng kg^{-1}. In the current study, the detected concentrations of AFM_1 in the examined drinking yoghurt samples were similar to the recorded findings by Heshmati *et al*.[54].

The low AFM_1 level (<50 ng kg^{-1}) in the examined flavoured drinking yoghurt may be attributed to the fermentation of drinking yoghurt, the reduced AFM_1 concentration in the used milk and also the companies which manufactured this product use the verified raw milk for AFM_1 levels[55].

CONCLUSION

The prevention of dairy animals from consuming contaminated feed with AFB_1 is the perfect factor for gaining free milk and their products from AFM_1 contamination. Besides, hygienic processing, ripening and storage shall be applied through the manufacturing and handling of milk derivatives for the prevention of aflatoxins' health risks.

Whereas the calculated EDI and HI for aflatoxin M_1 represent a moderate toxic risk for Egyptian kareish cheese consumers. Simultaneously, alertness shall be offered to periodical monitoring of these toxins in feeds and milk products. Plus, the governmental agencies should aware of the farmers, owners of dairy companies and these milk products consumers on the bad health effects of this toxin.

SIGNIFICANCE STATEMENTS

This study revealed a significant contamination level of Egyptian kareish cheese with Aflatoxin M_1 and calculated EDI and HI for aflatoxin M_1 represent a moderate toxic risk for Egyptian kareish cheese consumers. Subsequently, farmers, dairy plants and consumers should have general knowledge about the potential risk of this toxin. Protecting the dairy animals from eating contaminated feed with AFB_1 is the best way to prevent the generation of aflatoxin M_1 in milk and dairy products.

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