Quantitative Studies on Aflatoxin M1 Residues in Milk and Some Dairy Products

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Abstract

A total of hundred and forty samples of milk and dairy products (twenty each) of raw milk, UHT milk, set yoghurt produced at small and large scale, Damietta cheese, white soft cheese, and butter; were collected from different markets at Damietta Governorate during the period from October 2020 to March 2021 and examined for the presence of aflatoxin M1(AFM1) residues. The obtained results showed that all examined samples were contaminated with AFM1 but within the permissible limits recommended by Egyptian Standards and European Commission. The mean value for the AFM1 residues were 25.09 ± 1.7; 14.7 ± 0.35; 18.8 ± 0.2; 19.06 ± 0.7; 21.57 ± 1.28; 17.7 ± 0.32 and 16.69 ± 0.51 ng/L raw milk, UHT milk, set yoghurt produced at small and large scale, Damietta cheese, white soft cheese, and butter samples, respectively. The AFM1 has a considered health hazard even if present within permissible limits as it has an accumulative effect especially if it is present in a daily consuming food such as dairy products.

Keywords: AFM1, raw milk, UHT milk, yoghurt, Damietta cheese white soft cheese, butter, ELISA.

Introduction

Milk and milk products have high nutritional values and are considered as a daily balanced diet for infants and elders, thus it may be the main entrance route of aflatoxins to the human body ***(Maggira et al., 2021).***

Aflatoxins are toxins produced by molds mainly *Aspergillus flavus*, which produce Aflatoxin B, also *Aspergillus prasiticus* and *Aspergillus nomius* that produce both B and G aflatoxins ***(Zakaria et al., 2019)*** during growing on food with bad storage conditions ***(FAO, 2004).*** The aflatoxins have been categorized as group 1 human carcinogen and AFM1 as a group B carcinogen by the International Agency for Research on Cancer that mainly targets the liver

(IARC, 2002). Aflatoxin B1 (AFB1) is the most potent Aflatoxin which is biotransformed to Aflatoxin M1 (AFM1) by P450 hepatic cytochrome enzymes and secreted into milk, and there is a linear relationship between the amount of AFM1 in milk and AFB1 in feed consumed by animals ***(YEŞİL et al., 2019).*** AFM1 can be detected in milk within 12-24 hours after the first intake of AFB1 and it is relatively stable at high temperature and cannot be removed from milk by heat treatments, freezing, storage or, processing, and can transfer to milk products ***(Pecorelli et al., 2020)***.

Therefore, this study was designed to determine the concentrations of AFM1 in milk and some dairy products, collected from Damietta city, then compare the obtained results with the permissible limits specified by the Egyptian and European Commission as well as the public health hazards were discussed.

Materials and methods

1. Collection of samples: A total of 140 samples, 20 each of raw milk, UHT milk, small and large scale yoghurt, Damietta cheese, white soft cheese, and butter, were randomly collected from markets and dairy shops in Damietta Governorate, Egypt from October 2020 to March 2021 for detection of AFM1 residues.

2. Analysis of AFM1: collected samples were analyzed at the Microbial Toxin Unit, Animal Health Research Institute, Dokki. The quantitative analysis of Aflatoxin was performed according to the procedure described by ***R-Biopharm GmbH (1999).***

2.1. Materials and reagents: Commercial ELISA kits Biopharm RIDASCREEN® AFM1 (Art. No.: R1121).

2.2. Preparation of samples:

2.2.1. Preparation of milk samples: 10 ml of milk samples were centrifuged at 20°c and 3500 rpm/10 minutes. The cream layer was discarded and 100 µl of the defatted milk was used for the analysis.

2.2.2. Preparation of cheese samples: 5 gm of ground cheese were homogenized with 20 ml of methanol 70%, and warmed at 50°c for 30 minutes with periodical shaking vigorously then centrifuging at 3000 rpm/10 minutes at 10°c and filtrated. 2 ml of the filtrate transferred to 2 ml hexane then shaken and centrifuged at 3000 rpm /10 minutes. The lower aqueous phase was separated and diluted to 1:5 with buffer, then 100 µl per well was used.

2.2.3. Preparation of yoghurt samples: 2 gm of yoghurt samples were heated to 80°c for 3 minutes then cooled at room temperature.

The samples were diluted with 10 ml phosphate buffer solution (pH 7.2) then homogenized and 100 µl was used in the analysis.

2.2.4. Preparation of butter samples: 3 gm of butter samples were warmed at 40°c and centrifuged at 3000 rpm/minute at room temperature. The centrifuged butter was mixed with 3 ml of N-hexane and 3 ml of 70% methanol for 15 minutes then centrifuged at 4000 rpm/10 minutes at 10°c. 50 µl of the bottom liquid phase were diluted with buffer in a ratio of 1:17 then 100 µl of diluted samples were used for analysis

2.2.5. Test procedure: 100 µl of antibodies were added and kept at room temperature for 15 minutes. Plates were washed 3 times using 250 µl buffer and kept at room temperature for 30 minutes. 100 µl of prepared samples were washed with 250 µl buffer 3 times. 100 µl of substrate 1 chromogen was added to each well, thoroughly mixed, and kept for 30 minutes at room temperature in a dark place. Afterward, 100 µl stock solution was added to each well and thoroughly mixed then the result read at 450 nm with a pachet program.

2.2.6. Statistical analysis: The results were statistically analyzed by a one-way analysis of variance (ANOVA) using SPSS program version 26 **(SPSS, 2017)**. Probability levels of $p \leq 0.05$ were considered as a significant difference.

Results and discussion

The assessment in this study was carried out by using enzyme-linked immunosorbent assay "ELISA" which is a rapid and low-cost technique, thus it is the most used technique for AFM1 detection ***(Nejad et al., 2020)*.** The obtained results demonstrated in Table (1) showed that all examined samples were contaminated with AFM1 and within the permissible limits according to Egyptian Standards ***(EOSQ, 2010)*** and that legalized AFM1 concentration up to 50 ng/L for all samples, and according to European Commission ***(European Union, 2006)*** which allowed AFM1 limit 50 ng/L or kg for all samples except cheese samples which allowed 250 ng/kg.

AFM1 residues in dairy products can be due to three possible sources. First, AFM1 appears in raw milk after transformation of AFB1 come from contaminated feed. Second, AFM1 produced in contaminated dairy products after their production. Third, the presence of AFM1 in milk powder or other additives used during dairy product manufacture. Therefore, good manufacturing practice and storage is mandatory to prevent this hazard in dairy products ***(Mason et al., 2015).***

The mean of AFM1 in raw milk was 25.09 ± 1.7 ng/L which was nearly similar to those reported by ***Turkoglu and Keyvan, (2019)*** and higher than that estimated by ***(Amer and Ibrahim, 2010)*** *and* ***Ramadan et al., (2017)****,* but lower than those detected by ***Murshed, (2020)*** and ***Maggira et al., (2021).*** Regarding Aflatoxin M1 (AFM1) residues in UHT milk samples, the mean value was14.70 ± 0.35 ng/L that are nearly similar to those reported by ***Mohamadi and Alizadeh, (2010)*** *and* ***Turkoglu and Keyvan, (2019)****,* while ***Martins et al., (2007)*** detected lower AFM1 levels. Higher AFM1 concentrations were obtained by ***Erdemli Köse et al., (2019)*** *and* ***YEŞİL et al., (2019).*** AFM1 is a heat-stable toxin, thus it remains in heat-treated milk and dairy products, while some reported its decline after heat treatment up to 32% ***(Galvano et al., 2001).***

Concerning the mean of AFM1 levels in set yoghurt produced at small-scale and large-scale samples were 21.57 and 17.70 ng/kg, respectively. Nearly similar AFM1 concentrations in yoghurt were reported by ***Mason et al. (2015)*** and ***Farah Nadira et al. (2017).*** Higher concentrations were stated by ***(Iqbal and Asi, 2013)***, ***Tosun and Ayyildiz, (2013)*** *and* ***Murshed, (2020)***. In contrast, lower AFM1 levels were determined by ***(Galvano et al., 2001).*** The AFM1 in yoghurt could be lower than raw milk due to the presence of lactic acid bacteria resulting in acidic pH and organic acids production ***Murshed, (2020)***. Regarding AFM1 residues in cheese samples, the mean levels in Damietta cheese and white soft cheese were 18.8 ± 0.2 ng/kg and 19.06 ± 0.7 ng/kg, respectively.

These results are nearly similar to those reported by ***Yilmaz et al., (2018),*** meanwhile, ***Farah Nadira et al. (2017)*** detected lower AFM1 levels, whereas ***(Amer and Ibrahim, 2010)*** *and* ***Murshed (2020)*** recorded higher levels.

Concerning the AFM1 in butter samples, the mean was 16.69 ± 0.51 ng/kg that close to the findings reported by ***Bakirci (2001)*** while higher concentrations were detected by ***Iqbal and Asi, (2013)*** *and* ***YEŞİL et al., (2019).*** On the other hand, ***Fallah, (2010)*** estimated lower concentration and ***Tosun and Ayyildiz, (2013)*** didn't detect any AFM1 residues in examined butter samples.

The AFM1 has a great affinity toward casein so, it binds with curd during cheese formation, and with the broken fat globular membrane detached with whey during butter processing. Therefore cheese contains AFM1 more than milk, whey, cream, and butter in order ***(Pecorelli et al., 2020).***

The wide variation of AFM1 levels could be related to geographic and

climatic differences, feeding systems, and farm management ***(FAO, 2004).*** Moreover, the aflatoxins in milk produced in autumn and winter are higher than in spring and summer due to weather conditions and using stored forages that favor mold growth and mycotoxins production ***(Barukčić et al., 2018).*** Therefore**,** there is an economic impact including the cost of marketplace rejection and animal management which is more than $550 million per year in the US alone, and definitely, the global cost is much higher ***(Golge, 2014).***

The ***FAO/WHO, (2019)*** declared that there are about 6 billion consumers of milk and dairy products, demonstrating the significance of these products. Infants have higher body weight-based food intake and less developed detoxification abilities than adults, so they are more sensitive to such residues that may lead to growth retardation.

AFM1 is resisting greatly decomposition or digestion, and upon entering the body it may cause damage to the DNA leading to mutagenic and carcinogenic effects, and the main target organ is the liver ***(Saha Turna and Wu, 2021)***. The cytotoxicity and genotoxicity of AFM1 have been increasing lately especially after exposure to hepatitis viruses, particularly hepatitis B ***(FAO/WHO, 2019),*** and influenced by the duration and level of exposure which happens through frequent consumption of AFM1 and cumulating inside the consumer body**.** Therefore, it is important to pay attention to the AFM1 residues in food in general and milk and dairy products in particular even their levels were within permissible limits, as the result obtained in this study and as reported by ***Saha Turna and Wu (2021)*** who found that most countries around the world showed AFM1 levels below the EU limits. Simultaneously, milk and dairy products are daily consumed food which may raise the possibility of cumulated AFM1 in the body resulting in a higher incidence of its toxicity and carcinogenicity.

The LD50 for most animal species ranged from 0.5 to 10 mg/kg body weight ***(Dawit Akeberegn, 2019).*** However, the "Joint Expert Committee on Food Additives" (JECFA) of the World Health Organization and Food and Agriculture Organization didn't set tolerable daily intake (TDI) for any of the aflatoxins in humans, including AFM1 ***(JECFA, 2016)***. But, the Estimated Daily Intake (EDI) could be calculated through the following equation and expressed in ng kg $bw^{-1}day^{-1}$ ***(Maggira et al., 2021)***: **EDI** $= \frac{daily\ intake \times mean\ level\ of\ AFM1}{average\ body\ weight}$

The daily average consumption of milk for adults in each country is demonstrated by the Food and Agriculture Organization of the United Nations ***(FAOSTAT, 2017)*** which in Egypt is at least 0.113 kg/day and the amount in children is much higher. Presuming an adult body weight of 70 kg, and by using the previous equation and the obtained AFM1 in this study, the EDI would be at least 0.0404 ng kg $bw^{-1}day^{-1}$ which is higher than that obtained by ***(Amer and Ibrahim, 2010)***.

The Carcinogenic Potency (Pcancer) of AFM1 could be calculated as following: $Pcancer = 0.001 \times \%HBsAg^{-} + 0.03 \times \%HBsAg^{+}$ **(Udovicki *et al.*, 2019)**. According to the study carried out by ***Wasfi and Sadek (2011)*** the hepatitis B surface antigen [HBsAg] prevalence was 1.4% so, Pcancer for this study would be at least 0.1406 which means that 0.1406 cancers per year per 100, 000 population per ng Aflatoxins $kg^{-1}bw\ day^{-1}$. Furthermore the population risk could be estimated using this formula: Population risk = Exposure (EDI) × Average potency ***(Korley et al., 2021)*** as a result, the population risk in this study would be at least 0.0057.

Table (1) *Incidence and Concentration of AFM1 in Examined Samples (ng/L)*

Examined samples N= 20	Positive samples		The concentration of AFM1 (ng/L)			Samples confirmed the ES and EU	
	No	%	Min.	Max.	Mean ± SE	No.	%
Raw Milk	20	100.0	17.7	32.48	25.09 ± 1.7	20	100.0
UHT Milk	20	100.0	11.81	17.02	14.7 ± 0.35	20	100.0
Damietta Cheese	20	100.0	17.94	19.66	18.8 ± 0.2	20	100.0
White Soft Cheese	20	100.0	13.98	23.12	19.06 ± 0.7	20	100.0
Small Scale Yoghurt	20	100.0	16	27.13	21.57 ± 1.28	20	100.0
Large Scale Yoghurt	20	100.0	15.32	20.03	17.7 ± 0.32	20	100.0
Butter	20	100.0	13.52	21.1	16.69 ± 0.51	20	100.0

Conclusion and recommendations

The present investigation revealed that the examined raw milk, UHT milk, traditional Damietta cheese, white soft cheese, set yoghurt produced at small and large scale, and butter samples were contaminated with AFM1 but within the permissible Egyptian and European limits. To minimize the AFM1 residues in milk and dairy products, it is mandatory to prevent fungal growth in animal feedstuff through improving the processing and storage of feedstuff, as well as, using chemical aflatoxins inactivators in animal feed such as ammonia or Ozone gases. In addition, inform producers of the risk of aflatoxins and the ways to prevent its production, besides periodically checking the presence of AFM1 in milk and dairy products by food control agencies and implementing a food monitor system, such as HACCP system in dairy production .

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الملخص العربى

أجريت هذه الدراسة على 140 عينة من اللبن الخام و بعض منتجات الألبان المنتجة محلياً في أسواق مدينة دمياط (اللبن الخام – اللبن المعقم بالحرارة الفائقة – الزبادى المنتج على نطاق واسع وعلى نطاق ضيق – الجبن دمياطى التقليدي – الجبن الأبيض – الزبدة) و تم حفظ العينات بالطريقة الصحيحة و استبيان قيمة الأفلاتوكسين (إم1) قبل انتهاء صلاحية بعض هذه المنتجات باستخدام تقنية الإليزا (فحص انزيمات المناعة المرتبطة) . وبمقارنة التركيزات بالحدود المسموح بها عالمياً و محلياً اوضحت نتائج الدراسة ان كل العينات التي تم فحصها كانت تحتوي على الافلاتوكسين (إم1) و لكن بالحدود المسموح بها عالمياً و محلياً. و بالرغم من ذلك يجب التنويه عن ان متبقيات الافلاتوكسين (إم1) لها قدرة على التراكم داخل جسم المستهلك مسببة له مضاعفات خطيرة مما يشير الى اهمية الانتباه الى كميات الافلاتوكسين (إم1) التي يتم استهلاكها حتى لو كانت تحت القيم المسموح بها خاصة مع المنتجات التي تستهلك بصفة يومية مثل منتجات الالبان.