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RESEARCH ARTICLE

Effect of Storage on the Level of Aflatoxin M_1 in Milk and Other Dairy Products Sold at Tripoli Province, Libya

Elhadi Emh. Mohammad Gunbeaj[1], Syed Amir Ashraf[2], Nada Basher El-Akary[1], Subuhi Sherwani[3], Amir Mahgoub Awadelkareem[2], Wahid Ali Khan[4] and Mohd Wajid Ali Khan[3]*

[1]Department of Nutrition, College of Medical and Health Sciences-Al-Jumayl, Sabratha University, Sabratha, Libya. [2]Department of Clinical Nutrition, College of Applied Medical Sciences, University of Hail, Kingdom of Saudi Arabia. [3]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, University of Hail, Kingdom of Saudi Arabia. [4]Department of Clinical Biochemistry, College of Medicine, King Khalid University, Abha, Kingdom of Saudi Arabia.

Abstract

Milk and dairy products are one of the chief sources of nutrition for human beings particularly for infant and children. Aflatoxin M_1 (AFM_1) a hydroxylated metabolite of aflatoxin B_1 found in milk and milk products causes serious health issues for human beings. The objective of this study was to evaluate the effect of storage on the level of aflatoxin M_1 in milk and other dairy products sold at retail stores of Tripoli Province, Libya. Selected samples (Skimmed and cream milk, infant milk formula, butter, cheese, Cheddar, spread and slice) were evaluated by using specialized RIDASCREEN AFM_1 competitive enzyme linked immune sorbent assay (ELISA) technique. Our investigation revealed that, the concentration of AFM_1 increased with the duration of storage. Furthermore, we found that the newly manufactured samples had very low concentration of AFM_1 and within the permitted range. Moreover, AFM_1 concentration in skimmed and cream milk having 6 month shelf life had 5.00 $ngkg^{-1}$ and 5.03 ngkg-^{1} respectively. Furthermore, both the expired skimmed and cream milk had AFM_1 concentrations 121.8 $ngkg^{-1}$ and 108.18 $ngkg^{-1}$, respectively. In addition to that, we found that the levels of AFM_1 in different dairy products varies with different shelf lives (12 and 1 month), such as cheddar (5.0 and 72.79 $ngkg^{-1}$), Spread (5.30 and 60.03 $ngkg^{-1}$), Slice (5.50 and 61.18 ngkg-1). Additionally, infant milk formula with shelf life of 24 months and expired samples had AFM_1 less than 5.00 $ngkg^{-1}$ and 60.8 $ngkg^{-1}$, respectively. Based on our investigation, we found that the presence AFM_1 in milk and milk products at high concentration may cause serious illness to consumers' health and the consequent economic losses.

Keywords: Mycotoxins, Aflatoxin M1, ELISA, milk and milk products, Shelf lives.

***Correspondence:** mw.khan@uoh.edu.sa; wajidkhan11@gmail.com; +966-582680441

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INTRODUCTION

In recent years, occurrence of food contamination has been increased raising serious issue concerning human health and safety (Negash, 2018; Ibrahim et al., 2018). Milk and other dairy products are the key source of human nutrition especially for children across the globe (Tahoun et al., 2017). However, natural food contaminants present in milk may casue risk to human health. Therefore, it is not astonishing that a significant consideration has been paid over the several years to improve quality of milk (Garbaj et al., 2013). The occurrence of aflatoxin in milk and other dairy product has been well known across the world dairy industries (Iqbal et al., 2015). However, occurrence of aflatoxin from these fungal species *Aspergillus flavus, Aspergillus nomius* and *Aspergillus parasiticus* are more prominent. In addition to that, *Aspergillus flavus* produces only aflatoxin B, while the other species produces both aflatoxins B and G (Abdolgader et al. 2017). Furthermore, aflatoxin B_1 (AFB_1) produces a hydroxylated metabolite aflatoxin M_1 (AFM_1), which is excreted in milk when lactating animals are nurtured with contaminated food. Consumption of feedstuff contaminated with aflatoxin B_1 during lactation in bovine causes production of AFM_1 (monohydroxy derivative of B_1), which is excreted in the bovine milk (Iha et al. 2013). Among the aflatoxin, aflatoxin B_1 is particularly the most toxic metabolite, when consumed by dairy animals. In addition, biotransformation of metabolites AFM_1 occurs at the hepatic level using microsomal cytochrome p450 (European Commission 2003). Subsequently AFM_1 excreted in animal milk used for human intake (Baskaya et al., 2006; Battacone et al., 2005). In addition to that, aflatoxins does not have flavor or smell, they are fluorescent under the ultraviolet light and they are resistant to high temperatures more than 320°C without fragmenting (Garmakhany et al., 2011). Moreover, pasteurization of milk diminishes the chance of infection caused by pathogenic bacteria, but thermal treatment does not control other contaminants such as aflatoxin (Omer, 2016). AFM_1 is reasonably stable in raw and processed milk products and cannot be destroyed by heat treatments (Iha et al., 2011). Aflatoxin has been suggested as highly toxic, carcinogenic, teratogenic and mutagenic compounds, and it is well known as a causative agent for human hepatic and extrahepatic carcinogenesis. It has been reported that, high levels of aflatoxin in the bloodstream depress the immune system, which could cause stunting growth of children as well as low immunity levels in patient with HIV and cancer (Villers, 2014). There are various published reports, suggesting mycotoxins are interrelated for the prevalence of cancer in human beings. Which includes such as aflatoxin, zearalenone, fumonisin, sterigmatocystin, ochratoxin and patulin, (Ostry et al., 2009; Stec et al., 2009). International Agency for Research on Cancer (IARC) included aflatoxin B_1 as main causative agent for carcinogenic effect however AFM_1 is included into secondary groups of carcinogenic compounds (Ahmad et al., 2011; Kamkar et al., 2011). The European Community and Codex Alimentarius recommended tolerance range of AFM_1 in liquid milk and dried or processed milk foodstuffs should not exceed more than 50 $ngkg^{-1}$. However, as per the guideline from US, the permitted range of AFM_1 in milk should not go beyond 500 $ngkg^{-1}$. In Austria and Switzerland, the maximum levels are further reduced to 10 $ngkg^{-1}$ for infant food commodities (Abdolgader et al., 2017). However, we found that detection of AFM_1 with respect to shelf life of the milk and other dairy products have not been previously studied.

The objective of this study was to assess the Aflatoxin M_1 in milk and other dairy products having different shelf lives to make sure the safety of milk and dairy products that's been used in the markets of Libya as milk and dairy products are major source of energy in diet and consumed in great amounts yearly.

MATERIALS AND METHODS

Chemicals and standard solutions

AFM_1 reference standard (*Aspergillus flavus*, 10μg, A6428) was purchased from Sigma–Aldrich (Germany). Dichloromethane, methanol, PBS (Phosphate Buffered Saline), heptane, and deionized water of HPLC grade were purchased from Baker (Deventer, Netherlands).

Sample collection

A total of 21 samples were obtained (3 samples of skimmed milk, 3 samples of cream, 6 samples of Infant milk formula, 3 samples of Cheddar and 3 samples of spread and 3 samples of slice and 3 samples of butter) randomly from

the local markets of Tripoli, Libya and aflatoxin concentrations were determined by using enzyme linked immunosorband assay, ELISA (Ridascreen, aflatoxin M_1).

Sample preparation

Liquid milk

Degreasing of milk samples were performed by centrifuging at 3500g for 10 minutes and temperature was regulated at (10°C or 50 °F). Later on, the upper cream layer was distanted completely by aspirating through a Pasteur pipette. The skimmed milk was directly used for the experiment (100 µl per well) (Montagna et al., 2008).

Milk powder

10 g of milk powder weighed into a flask and 100 ml deionizer water were added and stirred for 5 min. Rest of sample preparation were performed as of milk preparation.

Cheese and Butter

The samples preparations were performed as mentioned above with slight modifications. A representative sample was chopped into coarse slice and later on thoroughly mixed, without the addition of liquid. 2g thoroughly mixed cheese weighed and placed into a centrifugal glass vial. 40 ml dichloromethane were added and extraction was done by stirring for 15 min. In addition to that, samples were filtered and suspension evaporated at 60 °C under nitrogen stream. After that, oily residues were re-dissolved with 0.5 ml methanol, 0.5 ml PBS buffer and 1 ml heptane. Later on, samples were mixed and centrifuged for 15 minutes at 2700 g at 15 °C. The upper layer of heptane was removed completely. Poured off an aliquot of the lower methanolic-aqueous phase carefully using a Pasteur pipette 100 µl of this aliquot diluted with 400 µl buffer 1 (1:5 dilution) and 100 µl per well are used in the test (Tihrumala-Devi, et al., 2002).

ELISA Analysis

To analyze the prepared samples, Ridascreen AFM_1 competitive enzyme immunoassay techniques were used for the estimation of AFM_1 in milk and other dairy products. Moreover, for estimation of AFM_1 required number of microtiter wells was inserted into the micro well holder for all standards and samples to be run in duplicate. Standards and samples position was recorded. 100µl AFM_1 standard solutions or prepared sample were added to separate duplicate wells and analyzed as per the instruction mentioned over the ELISA kit. Analysis of AFM_1 was performed using ELISA plate reader (Thermo Labsystems Multiskan Spektrum, 1500) at 450 nm against blank (Montagna et al., 2008).

Statistical Analysis

All the experiments were carried out in triplicates. Results were expressed as mean ± SD of three independent experiments (n = 3)

RESULTS AND DISCUSSION

In this study we tested the presence of AFM_1 in milk and dairy products by using specific ELISA (RIDASCREEN®) AFM1 30/15 technique used for AFM_1. Skimmed and creamed milk samples were investigated for AFM_1 with different duration of expiry dates (6 months, 1 months and expired) (Table 1). Skimmed and creamed milk sample whose expiry dates was 6 months did not show high level of AFM_1 (5 and 5.03 ngkg-1, respectively). Although, high values of AFM_1, recorded, but under threshold level (50 $ngkg^{-1}$) for both skimmed (33.9 $ngkg^{-1}$) and creamed (29.1 $ngkg^{-1}$) milk samples having expiry of 1 month. However, both expired samples of skimmed (121.8 $ngkg^{-1}$) and creamed (108.18 $ngkg^{-1}$) exhibited high amount of AFM1 that were more than the permissible limit. Both skimmed and creamed milk samples that were already expired exhibited maximum concentration of AFM_1 that is much higher than the permissible international value (50 $ngkg^{-1}$) for AFM_1 (European Codex 2000). Moreover, expired skimmed milk samples showed

Table 1. Level of aflatoxin M_1 in skimmed and creamed milk samples having different duration of expiry

Sample	Shelf life (months)*	Aflatoxin M_1 ($ngkg^{-1}$)
Skimmed*	6	5.00±0.05
	1	40.7±2.24
	Expired	121.5±5.52
Cream milk*	6	5.03±0.12
	1	28.1±1.17
	Expired	108.2±4.47

Values were expressed as mean ± SD
*Number of replicates for each sample; *n=3*

more concentration of AFM_1 as compared to the expired creamed milk samples. However, AFM_1 has been found to be unaffected to thermal energy, pasteurization, autoclaving and other varieties of food processing procedures (Boudra *et al.*, 2007; Hussain *et al.*, 2008). Value of 0.05 μgkg^{-1} of milk is currently the legal limit in those European countries that have the most stringent regulations for AFM1. This value was proposed by the Codex Committee on food additives and contaminants in 2000 (European Codex 2000). Therefore, it is important to stringent screening and routine-monitoring of AFM_1 levels in milk samples. However, it has been reported that due to high toxicity and carcinogenic properties of AFM_1, its presence in milk has been put consumer health on alarm.

Newly born and infants are highly dependent on infant milk formulas constitute an important or often sole source of food for them during the first months of life. However, these milk products are often given to the infants up to the age of 2 years. Moreover, AFM_1 levels in infant milk formula were presented in table 2. Which showed that, out of 6 samples of infant milk powder with different duration of expiry, only expired sample showed high concentration of AFM_1 i.e.; >60.18 ng kg-1. However, there were no significant differences (5 - 5.09 ng kg-1) observed in concentrations of AFM_1 in other 5 samples with 24, 12, 6, 3 and 1 month(s) to expire. Milk powder has been a frequently marketed product in retail stores during the recent years. It is used in chocolate, ice-cream, yoghurt, cake and baby food. Although infant milk powder has long duration of expiry dates, the samples with 24, 12, 6, 3 and 1 month gave less amounts of AFM_1. The samples from expired milk powder showed high concentration of AFM_1. From this we interpreted that there was no risk to give milk powder to babies until it was expired but it would be very harmful to the babies if unknowing expired mild given to them, as AFM_1 work as an immunosuppressant and babies have weak immune system and are therefore more prone to the diseases (Akhtar et al., 2017).

The widespread presences of aflatoxin M1 in cheese may be considered to be potential hazards for human health and that is why it is also important to identify the amount of AFM1 in various cheese (cheddar, spread and slice) available in the retail markets. Different cheese such as cheddar, spread and slice were also

Table 2. Level of aflatoxin M1 in infant milk powder with different duration of expiry

Sample	Shelf life (months)*					
	24	12	6	3	1	Expired
Aflatoxin M1 (ng kg^{-1})	<5.00±0.13	<5.80±0.15	<5.00±0.11	<5.03±0.18	<5.90±0.17	60.8±3.9

Values were expressed as mean ± SD; *Number of replicates for each sample; *n=3*

Table 3. Level of aflatoxin M1 in infant milk powder with different duration of expiry

Sample	Shelf life (months)*	Aflatoxin M1 ($ngkg^{-1}$)
Cheddar	12	5.00±0.13
Cheddar	1	72.79±4.2
Spread	12	5.30±0.70
Spread	1	60.03±2.9
Slice	12	5.50±0.22
Slice	1	61.18±4.5

Values were expressed as mean ± SD
* Number of replicates for each sample; *n=3*

Table 4. Level of aflatoxin M1 in butter with different duration of expiry

Sample	Shelf life (months)*	Aflatoxin M1 (ng kg^{-1})
Butter	12	5.00±0.19
	1	18±0.88
	Expired	88.2±2.84

Values were expressed as mean ± SD
*Number of replicates for each sample; *n=3*

investigated and values presented in table 3. Samples from cheddar, spread and slice having 12 month of expiry showed similar values of AFM1 i.e.; (5.0, 5.3 and 5.5 $ngkg^{-1}$, respectively). However, cheese samples from cheddar, spread and slice having 1month shelf lives showed higher amount of AFM_1 72.79, 60.03 and 61.18 $ngkg^{-1}$ respectively. The values were higher than the permissible limits (Ishikawa et al., 2016).

In addition to that, level of AFM_1 in butter sample showed gradual increase in residual concentration as shown in table 4. Maximum amount (88.18 $ngkg^{-1}$) of AFM_1 was detected in expired sample of butter. Previous studies showed that AFM_1 concentration in butter was lesser than in the milk from which it was made. This study was also supported by our results for the presence of AFM_1 in butter. Only expired sample of butter contains toxic level of AFM_1.

CONCLUSION

Aflatoxins are among the most toxic contaminant produced by *Aspergillus* species. Based upon our result, we found that the concentration of AFM_1 was increasing with the increase in duration of storage of sample. Freshly prepared samples had insignificant amount of AFM_1 and was within the limit of European Regulations. However, incidence of AFM_1 in milk and other dairy products is a serious health issue and must be monitored as well as prophylactic measures should be taken to prevent factors responsible for AFM_1 toxin production. We found that, it is very unsafe to use any dairy product and milk that is near to expiry or expired. We are regularly using milk in our daily life. In addition to that, this is high time to screen milk and other dairy products for the presence of toxic AFM_1 in Libya and set permissible levels for such products.

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CONFLICT OF INTEREST

The authors do not have any conflict of interest.

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