Evaluation of aflatoxin M1 in raw, processed milk and some milk products in Cairo with special reference to its recovery

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Abstract: In this study a screening survey was undertaken to determine the presence and levels of aflatoxin M1(AFM1) in locally produced dairy products. For this propose, a total of 141 dairy samples (raw milk, pasteursed milk, milk powder, yogurt and feta cheese)were analyzed to determined the level of AFM1 in these products.results obtained showed that AFM1 was found in 54.6% of milk and milk products samples. Lactic acid bacteria (Lactobacillus bulgaricus and streptococcus thermophilus) used for removal of AFM1in yogurt during manufacturing. It was found that Lactobacillus bulgaricus was of more binding ability than streptococcus thermophilus in reduction of AFM1, the results were discussed in details.
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1. Introduction

Aflatoxin M1 (AFM1) is the hydroxylated metabolite of aflatoxin B1 (AFB1) and can be found in milk and subsequently in other dairy products when lactating animals are fed with contaminated feedstuffs. Mammals that ingest aflatoxin B1 (AFB1)-contaminated diets excrete amounts of the principal 4-hydroxylated metabolite known as aflatoxin M1 into milk

Aflatoxins (AF) are a group of highly toxic secondary metabolic products of some Aspergillus spp.; they easily occur on feeds and foods during growth, harvest, or storage. Aflatoxins are actually toxic, immunosuppressive, mutagenic, teratogenic and carcinogenic compounds to animals and humans, and contamination of feed and food is a current problem (Piva, *et al*,1995 Kotsonis,*et al*., 1996; Peraica, *et al*., 1999; Kocabas & Sekerel, 2003)

The presence of AFM1 has to be considered a risk, and never should be underestimated or neglected due to the unpredictability of climatic and environmental conditions and the inability of certain agricultural systems (characterized by poor economic conditions and/or lack of knowledge) to face and manage mycotoxin prevention/ contamination. The occurrence of AFM1 in milk, espe cially cow's milk, makes it a particular risk for humans because of its importance as a food stuff for adults and children.

Aflatoxin M1 is the principle hydroxylated metabolite of aflatoxin B1 which is transformed at the hepatic level by means of cytochrome P450 enzymes and excreted into the milk in the mammary glands of both human and lactating animal after ingestion by the animal of pellets and forage contaminated with aflatoxin B1 (Oveisi, Jannat, Sadeghi, Hajimahmoodi, & Nikzad, 2007; Prandini et al., 2009).

The best way to control the presence of AFB in food and feeds is to prevent their formation. Various physical, chemical and biological agents have been used to detoxify aflatoxins from food and feed materials (Nkana, 1987; Park, 1993). But there are currently no acceptable methods to counteract the AFM1 occurrence in milk and dairy products. Thus, a practical and effective method is needed to be developed for the detoxification of AFM1 contaminated milk or decreased its toxsity. Some strains of lactic acid bacteria have been reported to be effective in removing AFB1 and AFM1 from contaminated liquid media and milk (El-Nezami *et al.*, 1998; Hwang, *et al.,* 2005; Lee *et al*., 2003; Peltonen, *et al*.,2001; Zinedine *et al*, 2005; Shahin, 2007).

ELISA (Enzyme-Linked Immunosorbent Assay) method was used for measurement of AFM1. This method is established as a high throughput assay with low sample volume requirements, and often has less sample clean e up procedures compared to HPLC method (ISO, 2002). Due to the lack in data about the natural occurrence of AFM1 in Egyptian milk and other dairy products, this work was accomplished to clarify the

actual AFM1 occurrence in some of these products

In Egypt little available data, if any, was found about the occurrence of AFM1 in milk and milk derivatives. SoThe aim of the present study was to investigate the occurrence of AFM1 in raw, pasteurized , powdered milk ,yogurt and feta cheese available in Cairo using a 96-well microtitre plates ELISA test kits for AFM1 determination in addition, investigate the ability of some strains of lactic acid bacteria (Lactobacillus bulgaricus and Streptococcus thermophilus) used by the local dairy industry to remove this mycotoxin from contaminated milk.

2.Materials and methods

A total of 141 samples of commercial Egyptian milk and milk products were purchased from different different localties in Cairo supermarkets.

Milk samples

A total number of 141 samples were collected from different Cairo regions and markets during 5 months and were then evaluated for the presence of AFM1. Collected raw milk samples were transported to the laboratory in ice box and stored at —20 °C while being protected from light until the time for analysis.

Raw milk

Forty eight raw milk samples were collected. Samples were from different local small and large dairy shopes from various regions of Cairo.

Pasteurized milk

Thirty seven samples of commercial pasteurized cow milk were purchased from supermarkets in Cairo. Samples were analyzed before their expiry date.

Milk powder

Ten grams of milk powder was placed in a flask and 100 ml of deionized water was added. The mixture was stirred for 5 min then centrifuged at 3500 g and 20 °C for 10 min. After centrifugation, the upper fatty layer was removed, and 100 µl of the skimmed milk was used in the ELISA determination ofAFM 1 as described in the section on.

Yogurt samples

Twinty two yogurt samples were prepared according to the method outlined in the ELISA kit for cheese samples. Two grams of a representative samples were added to 40 ml of dichloro methane. The mixture was extracted by shaking for 15 min. Suspension was filtered and 10 ml of the filtrate were evaporated at 60 °C under weak N2 gas stream. The oily residue was redissolved in 0.5 ml methanol, 0.5 ml PBS buffer and 1 ml hexane. The mixture was centrifuged at 2700 g and 15 °C for 15 min. The upper layer of hexane was removed and 100 µl of the aliquot were diluted with 400 µl of kit buffer. Hundred µl of the diluted samples was used for the AFM1 measurement.

Feta cheese samples

fifteen Samples of Feta cheese were analyzed for the presence of AFM1. Samples randomly derived from different supermarkets of Feta cheese . Five samples (one sample from each unit) were performed on a weekly basis from May 2003 to February 2004, as described in Table 1. A total of 15 Feta cheese samples were analyzed. Analysis of samples carried out in less than 24 h from the time of their arrival in the laboratory.

Determination of AFM1 content :

The quantitative analysis to evaluate the incidence of AFM1 in the different milk samples was performed by competitive enzyme immunoassay using Ridascreen® AFM1 kits (R-Biopharm, Dermstadt, Germany), which contained microtiter plates coated with specific antibodies to AFM1, AFM1 standard solution of (0, 5,10, 20, 40, and 80 ng/l), peroxidase conjugated AFM1, together with substrate/chromogen and stop solution.

Preparation of milk samples :

Liquid milk.

For raw and pasteurized milk, 20 mL of milk were chilled to 10° C and was centrifuged for 10 min at 3500 g. The fatty layer was removed and 100 µL of the defatted milk was applied directly in the ELISA microtiter plate.

Milk powder.

Ninteen samples of milk powder (Ten grams ofpowder milk was placed in a flask, and 100 mL ofdeionized water was added. The mixture was stirred for 5 min and then centrifuged at 3500 g for 10 min at 10°C temperature. After centrifugation, the upper fatty layer was removed and 100 µL of the skimmed milk was used for ELISA analysis).

AFM1 analysis in dairy samples :

The method used in mycotoxin detection was the enzyme-linked immunosorbent assay (ELISA). ELISA kits were purchased from "Biopharm": RIDAScreen aflatoxin M1 (Cat. No. R1101) with a detection limit at 0.005 µg/l and recovery rates of 98% and 95% for milk and yogurt, respectively. All milk and yogurt samples were prepared following the method outlined in the ELISA kits.

Absorbance values: The mean values of the absorbance values obtained for the standards and the analyzed samples were divided by the absorbance value of the first standard (zero standard) and multiplied by 100. The zero standard is thus made equal to 100% and the absorbance values are quoted in percentages

A sufficient number of microtiter wells were inserted

into the microwell holder according to the manufacturer's instructions. One hundred microliter of the AFM1 standard solutions and of the samples (100 µL/well) were added in duplicates to the wells and incubated for 30 min at room temperature in the dark. The wells were washed three times with 250 µL washing buffer. After the washing steps, 100 µL of the peroxidase conjugated AFM1 were added and incubated for 15 min at room temperature in the dark. After incubation, wells were washed again for three times with 250 µL washing buffer, and one 100 µL of substrate/chromogen were added to each well, gently shaking the plate and incubated for 15 min at room temperature in the dark. At the end of incubation

AFM1 removal study:

Preparation of lactic acid bacteria (LAB) inoculums :

Lactobacillus bulgaricus and Streptococcus thermophilus strains were obtained from a Egyptian dairy industry. Lactobacillus bulgaricus was cultivated in 25 ml MRS broth (Oxoid CM 359) at 37 °C for 24 h and Streptococcus thermophilus was cultivated in 25 ml M17 broth (Oxoid CM817) at 37 °C for 24 h. Bacterial samples was taken every 2 h to determine the state of growth using a Neubauer haemocytometer until showed steady growth is observed. Thereafter, each bacterial inoculum was adjusted and maintained at 1 x 10^6 cells/ml.

Binding ability of LAB in AFM1 contaminated PBS medium :

In order to study the binding ability of LAB, 1 mL of each pure culture was suspended in a Falcon tube containing 49 ml PBS contaminated with AFM1 at a concentration of 50 ng/l and incubated at 37 °C for 14 h. During the incubation period, 2 mL of each bacterial suspension (pure cultures) were taken every 2 h and centrifuged at 3500 x g for 10 min. Unbound AFM1 content in the supernatant was determined by ELISA. For the ELISA analysis, samples were performed according to R-biopharm GmbH procedure. Cell-free PBS contaminated with AFM1 was used as positive control. Bacteria suspended in non-contaminated PBS were used as negative control (pure species) and for bacterial growth (pure species) during the assay time.

3.1.Results and disscusion:

The results of our study revealed that AFM1 was found in 54.6 % of tested milk product samples. Among the 141 analyzed samples, 64 samples (46.4 %) did not reveal the presence of this toxines (below the detection limit). Fourty eight samples of raw milk 37 of them were positive with percentage of 77.0%, the range of AFM1 WAS 3.41-137 ng/l . respecting to pasteurized milk 37 samples 8 of them were positive with percentage 21.6% . AFM1rang was 6.28-67.4 ng/l. Concerning to milk powder the study analysed 19 samples ,5 of them were positive with percentage 26.3% AFM1 range was 2.15- 16.5 wkile sampled of yogurt were 22 samples17 were positive withpercentage 77.6% AFM1 ranged from 9.70 to 89.3. in addition, Feta cheese samples were 15 sample 10 of them were positive with percentage 66.7% AFM1 range was 7.14-122 ng/l Table 1.

Global contamination of milk and dairy products by AFM1 was the subject of numerous studies. Due to the lack in data about the natural occurrence of AFM1 in Egyptian milk and other dairy products, this work was accomplished to clarify the actual AFM1 occurrence in some of these products. In order to discuss our results, it would be useful to compare the obtained AFM1 incidence and levels of contamination to those reported in other countries. For example, Garrido, Iha, Santos Ortolani, and Duarte Favaro (2003) found an incidence of AFM1 contamination of 73.4% (58 positive samples of 79) in pasteurized milk in Sao Paulo, Brazil. In their study, 26.6% of the samples (21/79) contained no detectable levels of AFM1, 58.2% (46/79) contained AFM1 at levels between 0.015 and 0.05 mg/l, and 15.2% (12/79) had levels between 0.05 and 0.5 mg/l. In Italy, Galvano et al. (2001) found the incidence of AFM1 contamination to be 78% (125 positive samples per 161 samples analyzed). However, none of the positive samples contained levels above 0.05 mg/l. A survey in Portugal reported that the incidence of AFM1 in raw milk and UHT milk was 80.6% and 84.2%, with concentrations ranging 0.005-0.061 ng/l (Martins & Martins, 2004). In Iran, 128 milk samples were analyzed and AFM1 was detected in 99 samples (78%) that were contaminated with a high level above the European limit (Oveisi, Jannat, Sadeghi, Hajimahmoodi, & Nikzad, 2007). In Syria, among 126 milk samples tested 80% were contaminated with AFM1 and 53 o f the positive samples (52.5%) contained levels that exceeded EC regulations (Ghanem & Orfi, 2009). In addition, studies conducted in Greece indicated that levels of AFM1 in milk were very close to the maximum tolerated dose (Kaniou-Grigoriadou, Eleftheriadou, Mouratidou, & Katikou, 2005).

Table 1 : showing occurance of AFM1 in milk samples and some dairy products.

Milk products	Numberof sample	AFM1 ranging (ng/l)	Positive samples	Negative samples	Percentage %
Raw milk	48	3.41-137	37	11	77.0%
Pasteurized milk	37	6.28-67.4	8	29	21.6%

Milk powder	19	2.15-16.5	5	14	26.3%
yogurt	22	9.70-89.3	17	5	77.2%
Feta cheese	15	7.14-122	10	5	66.7%
total	141	2.15-137	77	64	54.6

With respect to yogurt, Galvano et al. (1998) showed that in 114 yogurt samples, 91 (80.0%) contained AFM1 at levels ranging from 0.001 to 0.496㎎/l. Later, Galvano et al. (2001) reported that in 120 yogurts samples, 73 (61.0%) were contaminated with AFM1 at lower levels (0.001-0.32㎎/l) than those tested in the previous survey of 1998. In Portugal, 48 samples of yogurt were tested and only 2 (4.2%) contain AFM1 at levels of 0.045㎎/l (Martins & Martins, 2004). In Brazil; Sylos, Rodriguez-Amaya, and Carvalho (1996) did not detect the presence of AFM1 in 30 of tested yogurts. However, in a survey carried by Kim et al. (2000) in Seoul, South Korea, the presence of AFM1 was detected in 50%

Concerning to feta cheese, the samples of 15 the examined samples, 66.7% contaminated with AFM1, in the range of 7.14-122 ng/l, and were not found to have similar levels of AFM1 (97.5%) in comparison to Parvaneh study. Parvaneh *et al*., (1982) showed that of a total 80 cheese samples, 26.5% contained AFM1 in the range of 10–250 lg/kg, whilst 2.5% samples were not found to be contaminated with AFM1. We observed higher levels of AFM1 than the limits (0.25 lg/kg) set by some of the countries (such as Turkey) in 60.6% Feta cheese samples.

In many studies, the levels of contamination of cheese by AFM1 seem to vary . These variable results may be explained in part by different reason such as cheese manufacturing procedures, different milk contamination, type of cheese, condition of cheese ripening, and the analytical methods employed (McKinney *et al*., 1973; Kiermeier & Mashaley, 1977 Wiseman & Marth, 1983; Galvano *et al*., 1996)

3.2. Efficiency of AFM1 removal by lactic acid bacteria

In vitro binding ability of AFM1 by Lactobacillus bulgaricus and Streptococcus thermophilus was investigated in PBS liquid medium and during yogurt making comparatively. AFM1 removal was evaluated every 2h for 10 h in PBS assay during yogurt production. Lactobacillus bulgaricus showed high percentage of AFM1 binding ability (87.6% after 10 h) compared to Streptococcus thermophilus (70% after 10 h) in PBS (table 2).which showed the presence of free AFM1 in PBS medium before binding with LAB. In relation to the time with interval 2 h. Lacto bacillus bulgaricus showed high percentage of binding ability 60 % after 2 h., 50 % after 4 h, 42% after 6 h., 33% after 8 h. And 19% after 10 h. It was clear that with the time lactic acid bacteria showed inceased ability of binding to AFM1 while, streptococcus thermophilus showed less effecicy in binding ability to AFM1 comaring to control (table 2).

Table 2: in vitro presence of free AFM1before binding by LAB.

Groups	2h	4h	6h	8h	10h
Lacto.bulgarius	60%	50%	42%	33%	19%
Strept.therophilus	78%	69%	63%	42%	31%
Control	83%	87%	93%	100%	100%

This is in agreement with other studies on aflatoxins removal by lactic acid bacteria (El-Nezami et al., 1998), where Lb. rhamnosus strains GG and Lc 705 bound approximately 80% AFB1 within 0 h. Moreover, Turbic, Ahokas,Haskard (2002) showed that 77-99% AFB1 were removed by Lb. rhamnosus strains in high and moderate amounts and El Khoury *et al.*, (2011) who showed that the lactic acid bacteria (Lactobacillus bulgaricus and streptococcus thermophilus) used in the Lebanese traditional industryhave the ability to bind AFM1 with percentage (87.6%) and (70%) after 14 h. respectively.

The mechanism of aflatoxins removal by Lactic acid bacteria is still unknown, it has been suggested that aflatoxins molecules are bound to bacterial cell wall components rather than metabolically degraded (El-Nezami *et al.,* 1998; ; Haskard *et al*.,2001; Lahtinen *et al.,* 2004; El Khoury *et al*., 2011) suggested that AFB1 is bound to bacteria through weak non-covalent interactions such as association with hydrophobic pockets on the bacterial surface. The differences in AFM1 binding by the strains are probably due to different bacterial cell wall and cell envelope structures.

The binding ability of AFM1 by Lactobacillus bulgaricus and Streptococcus thermophilus was investigated during the making of yogurt . When LAB cultures in PBS and skimmed processing milk were compared, the binding was much greater in milk. The principal reason of that is may be due to the binding properties of AFM1 to milk casein. Brackett and Marth (1982) reported that

an average of 30.7% more AFM1 was found in milk once treated with proteolytic enzyme than in untreated milk and suggested that AFM1 is bound to milk protein. This is in agreement with our study where AFM1 binding by Lactobacillus bulgaricus in yogurt was 40 % after 2 h of incubation compared to 17 % in PBS medium (contro) . The binding level of AFM1 by Lactobacillus bulgaricus increased with time to reach 68% after 6 h of incubation. As expected Streptococcus thermophilus showed lower binding ability (22% removal after 2 h and 37% after 6 h) during yogurt making in comparison to Lactobacillus bulgaricus .

The current findings showed that the level of AFM1 contamination in Egyptian milk , yogurt and feta cheese is relatively lower to that observed in other regions around the world; those results may be explained by the fact of the low AFB1 contamination of raw feed and the long grazing period of cows during the year. This is of great importance, because low levels of AFM1 in milk may contribute to a decrease in the overall ingestion of aflatoxins through the diet.

4. Conclusion

AFM1 in Cairo dairy products comparing to other regions worldwide,was low level. the current results in this study imply that more emphasis should be given to the determination of AFM1 levels in Cairo milk and derived dairy products. Also, lactic acid bacteria, LAB (Lactobacillus bulgaricus and Streptococcus thermophilus strains) , used in dairy industries were shown effective in reduction the extent of free AFM1 content in liquid culture medium. Therefore, LAB seem to play a role in AFM1 removal and could be used as a biological agent for AFM1 reduction.

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