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ORIGINAL ARTICLE

Rapid detection of aflatoxin M1 residues in market milk in Aswan Province, Egypt and effect of probiotics on its residues concentration

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ABSTRACT

Objectives: The objectives of our study were to determine the presence of Aflatoxin M1 (AFM1) in market milk in Aswan province, Egypt and studying the effect of addition of some strains of probiotics microorganisms on AFM1 level in milk.

Materials and Methods: Between July and October 2018, 90 market milk samples (15 Ultra Heat Treated (UHT) , 75 raw) were collected from different dairy shops in Aswan City, Egypt to be examined for AFM1 presence by rapid strip test and the results were confirmed by high-performance liquid chromatography (HPLC).

Results: The results revealed that all UHT milk samples were negative, while 37 (49%) raw milk samples were positive for AFM1 residues. All 37 positive milk samples were examined by HPLC to determine the level of AFM1. The results showed that the level of AFM1 ranged between 0.053 and 0.207 with mean ± SE of 0.1003 ± 0.008 ppb. Some probiotics strains were used to determine their effect on AFM1 by milk fermentation; the result showed that the probiotics have significant effect on the reduction of AFM1 level in milk ($p < 0.05$). Also, Public health importance of AFM1 was discussed.

Conclusion: Presence of AFM1 in 49% of examined raw milk samples indicate widespread occurrence of AFM1 in market milk in Aswan province, Egypt which considered possible hazards for consumers, while the absence of AFM1 from UHT milk indicates that type of milk is safer. So, regular monitoring of AFM1 in market milk is necessary for evaluating their contamination status. Mixed starter culture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* could be used as a biological agent for the reduction of AFM1 in milk.

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Introduction

Incidences of milk contamination had been increased in the recent years which raised the question about the effect of different contaminants on economic consequences and public health [1]. Mycotoxins are one of the most serious food contaminants in the world. Aflatoxins (AFs) are a part of mycotoxins large family that including AFs, Fumonisins, Ochratoxins, Trichothecenes, Patulin, and Zearalenones. Animals are exposed to AFs by consumption of feeds contaminated with AF producing molds during storage, harvest, and/or growth [2]. When lactating animals fed on Aflatoxin B1 (AFB1) contaminated feed, AFB1 biotransformed in the liver into monohydroxy derivative AFM1 and expressed in their milk [3]. The occurrence of Aflatoxin M1 (AFM1) in commercially available milk is considered as a potential risk for public health because of frequent and prolonged exposure to a toxic and carcinogenic substance. AFM1 causes acute and chronic mycotoxicosis. Large doses of AFs lead to liver failure and death, while chronic

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sub-lethal doses have immunological and nutritional consequences and both doses have a cumulative effect that increases cancer risk [4]. *In vitro* AFM1 cause DNA damage, chromosomal anomalies, gene mutation, and cell transformation in mammalian cells [2]. AFM1 is one of the additional risk factors for Hepatocellular Carcinoma (HCC) development, through mutation in P53 tumor suppressor gene, and damage of DNA in liver cells. HCC is considered as the most common malignancy in the world [5]. Every year, incidence of HCC increased between 3% and 9%. In Egypt, about 4.7% of Chronic Liver Disease patients suffer from HCC. AFM1 is prevalent among cirrhotic patients in Egypt and showed the highest concentration in the serum and the urine among HCC patients from Upper Egypt compared to HCC patients of Delta Egypt [6]. Regarding male reproductive system, AFs consumption negatively affects spermatogenesis, spermatozoa fertility after ejaculation, cause infertility problems due to increased morphologic abnormalities of sperm cells, reduced sperm cells motility, count, and viability [7].

AFM1 is a heat stable compound, resistant to thermal treatments, such as pasteurization or sterilization, and is not degraded; therefore, its level in contaminated foods remains unaffected by heating methods and not destroyed during food processing [8]. So, prevention of AFB1contamination is the best way to control their presence in food. Various chemical, physical, and biological agents have been used to detoxify aflatoxins from food and feed materials. But, elimination of AFM1 from milk with physical and chemical methods have some disadvantages, such as losing its nutritional value, insufficiency of toxin elimination with high costs [9]. Thus, an effective and practical method is needed to be developed for the detoxification of AFM1 contaminated milk. Some strains of lactic acid bacteria can be used to detoxify or decrease toxins in milk. Such useful probiotics microorganisms are added to milk or dairy products at the different stages of processing and manufactures [4].

The efficient control of AFs residues in milk requires efficient detection which could be done by screening tests. The screening tests allow simultaneous analysis of large numbers of samples giving rapid results and inexpensive. The results should be confirmed by the quantitative method generally with chromatographic methods to determine the level of residues present [10].

Therefore, the objectives of our study were to determine the presence of AFM1 in market milk in Aswan province, Egypt by rapid methods and studying the effect of addition of some strains of probiotics microorganisms (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) on AFM1 level in milk.

Materials and Methods

All procedures were done according to the guide approved by the Ethics Committee of the Faculty of Veterinary Medicine, Aswan University, Egypt.

Samples collection

A total of 90 (15 Ultra Heat Treated (UHT) and 75 raw) randomly selected market milk samples were collected from dairy shops of Aswan Province, Egypt between May and October 2018 to estimate the presence of AFM1 residues. All samples were transferred to laboratory in ice box within 1 h and were frozen till the time of examination.

Detection of AFM1 residues in milk samples by quick AFM1 strip test kit

The New Quick AFM1 Strip Test kits were used in rapid qualitative/semi quantitative determination (10 min) of AFM1 in the milk samples. All that is needed comes with the kit; therefore, the test can be performed everywhere. Kits were purchased from [AstoriTecnicas.n.c., VAT Nr.: IT03112840172, Via Stelle, 11, Poncarale (BS), 25020, Italy/Italia].

Principle

The assay depends on a competitive colloidal gold-based format. It detects the presence of AFM1 at concentration of 0.05 ppb or higher in milk samples by utilizing specific reactions between antibodies and AFM1 in milk samples. The test is rapid, does not need to refrigerate or pre-heat milk samples, user-friendly, saves time, cost-effective, saves energy, saves milk, and long shelf life.

Procedures

The detection of AFM1 residues in milk was done by the methods described by the manufacturer.

Quantitative determination of AFM1 residues in milk samples by HPLC

High-performance liquid chromatography (HPLC) was used according to Choga et al. [11] to determine the level of AFM1 quick strip test positive milk samples.

Chromatographic condition

Flow rate 1 ml/min, Injection volume 20 µl, column temperature 50°C. UV-detector wave length: 233 nm. The mobile phase: methanol: water (97:3).

Method of validation

An external standard method was used for quantitative evaluations. Each sample was measured, and at the same

time, a blank sample per series was also measured, the milk samples were not containing AFM1 to test for the method selectivity. The standard curve was formed. Quantification of residues was obtained from area under curves and calculated automatically by the software (Fig. 1).

Effect of probiotics on the stability of AFM1

Lyophilized starter culture (YoFlex® Express 2.0Chr Hansen, Horsham, Denmark), containing *L. bulgaricus* and *S. thermophilus* that used in yogurt manufacture, was used to determine the effect of lactic acid fermentation on the AFM1 level.

Procedures

Ten raw milk samples contained AFM1 residues with different concentrations were pasteurized separately at 85°C for 5 min before being cooled to inoculation temperature (40: 42°C). After cooling, the starter was inoculated at a concentration of 1:1,000 and the mixtures were transferred to sterile plastic containers which were kept in incubator at 42°C for 4 h. The pH was measured by pH meter then fermented milk samples were kept in the refrigerator at 4°C for 1 day then examined by HPLC to determine the level of AFM1 after fermentation.

Statistical analysis

The data were presented in mean ± SE, and statistical analysis was carried out using SPSS program, version 18.0. A *t*-test was used for statistical analyses with repeated measures. *p* values < 0.05 were considered as significant.

Results and Discussion

Milk is an essential source for human nutrition so its safety and quality are of major importance, especially after continuous increase of the human population [4]. AFM1 is the most dangerous mycotoxin found in milk. Presence of AFM1 in milk represents a world health concern even if present in small amounts.

In Aswan province, Egypt, there is a lack of information about AFM1 residues in human foods so the present study was carried out to evaluate the occurrence of AFM1 in market milk in Aswan province by rapid methods.

Table 1 shows that the Rapid strip test was used for qualitative detection of AFM1 in 90 market milk samples (15 UHT and 75 Raw) and the results showed that all UHT milk samples were negative for AFM1 residues.

The negative results of our study for UHT milk samples are in contrast with that reported by Alborzi et al. [12], Marouf [13], Shaker and Elsharkawy [14], and Koack et al. [15] whom detected AFM1 in 38%, 96.4%, 100%, and 75.6% of examined UHT milk samples, respectively. The negative results of UHT milk samples did not mean that all the samples were free from AFM1 as there is a prospect that some samples might have AFM1 residues below the detection limit which is 0.05 ppb, thus the test could not detect it.

In the same time, regular monitoring of AFM1 in milk, good manufacturing practices, good storage practices, and implementing food safety programs, such as HACCP system in the dairy industries, may be the cause for low incidence of AFM1 presence in UHT milk [16].

In the current study, 37 (49%) raw milk samples were found positive for AFM1 residues with concentration ranged between 0.053 and 0.207 and mean ± SE 0.1003 ± 0.008 ppb (Tables 1 and 2).

Our result for some extent is similar to that reported by Amer and Ibrahim [17] and Aiad and Aboelmakarem [18] whom detected AFM1 in 38% and 40% of examined raw market milk with mean concentrations of 49.7 ± 17.3 ng/l and 8.30–85.00 ng/kg, respectively, in Alexandria, Egypt. In contrary, higher result were reported by Ghareeb et al. [19] and Jajic et al. [20] who detected AFM1 in 97.9% and 80.9% of examined raw milk samples with mean concentration of 62.9 ± 32.1 ng/l and 0.216 ± 0.470 µg/kg,

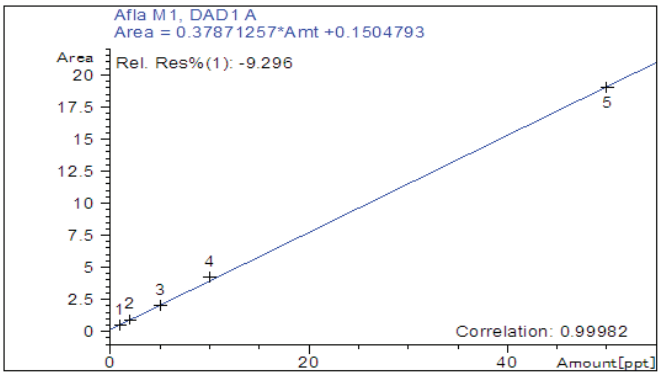

Figure 1. Calibration curve for AFM1.

Table 1. Incidence of AFM1 residues in Aswan market milk samples.

Types of milk	No. of examined milk samples	Positive samples	
		No	%
UHT	15	0	0
Raw	75	37	49
Total	90	37	41

Table 2. AFM1 concentration (ppb) in positive milk samples.

No of examined samples	Min	Max	Mean ± SE
37	0.053	0.207	0.1003 ± 0.008

respectively. AFs are secondary metabolites produced by *Aspergillus flavus and Aspergillus parasiticus* fungi when the moisture content exceeds 7% and the temperatures are between 24°C and 35°C [21]. The incidence and level of AFs are variable due to the source of AFs contamination of dairy foods. Variation in temperature and humidity conditions from country to country or from different areas at the same country affect the sources of mycotoxin contamination. Dry regions are less susceptible as compared to tropical and sub-tropical regions [22]. South Egypt has very hot summer and hot desert climate and very little precipitation year. Due to high temperature (39°C–41°C) and humidity (58%–61%), it can be predicted that the amount of AFB1 is high in the animal feeds. Increasing daily intake of AFB1 contaminated feed by dairy cattle will increase the incidence of AFM1 presence in milk [19].

The high stability, toxicity, carcinogenicity, and frequent presence of AFM1 in milk have prompted several countries to set up maximum residual limits (MRLs) for AFM1 in milk and its products to exclude the possible human toxicity. The Egyptian Ministry of Health recommended that milk and dairy products should be free from AFM1 residues [23], European Community [24], and Codex Alimentarius [25] recommended that the MRL of AFM1 in liquid or dried milk and processed milk products is 0.05 ppb, while Administration [26] established 0.5 ppb as a MRL for AFM1 residues in milk. From Table 2, we observed that all positive milk samples contain AFM1 concentrations exceeded Egyptian, Codex and EU standards, while all of them are within the US regulations. The same results were reported by Stoloff et al. [27] and Yıldırım et al. [28].

Reduction of milk contamination with AFM1 occurs either directly by decreasing the AFM1 content in contaminated milk or indirectly by decreasing AFB1 level in the feeds of dairy animals [29]. Well storage of the animal feed at the low temperature and dried area is recommended to prevent mold growth and AF production.

Probiotics are safe and cost-effective for human and animals to eliminate, detoxify AFs in milk or dairy products compared to other procedures, such as chemisorbents, antibodies, and even additives. Such useful microorganisms can be added to milk at the different stages of processing [4]. Lactic acid bacteria, *S. thermophilus* and *L. bulgaricus* are probiotics strains that were used in dairy industries and shown effective in the reduction of AFM1 during yogurt processing [30].

Table 3 revealed that there was a significant decrease ($p > 0.05$) in the mean levels of AFM1 before and after addition of probiotics. Therefore, the usage of mixed starter culture of *L. bulgaricus* and *S. thermophilus* in milk fermentation has beneficial effect on the reduction of AFM1 level.

The same results were reported by El Khoury et al. [30], Montaseri et al. [31], and Tahoun et al. [9]. On the other hand, Iha et al. [32] reported that the fermentation process of yoghurt manufacture has no effect on AFM1. It is known that the probiotics remove AFs by adhesion to bacterial cell wall rather than by metabolism or covalent bindings as dead and non-viable bacteria does not lose their adhesion ability [33,34]. Probiotics also have many advantages that decrease the adverse effect of AFs on human heath as they possess anti-carcinogenic activity, support the immune system, decrease populations of gastrointestinal pathogens, maintain normal intestinal flora when receiving antibiotics treatment, induce the pollen allergens resistance and protect lipids, DNA and proteins against oxidative damages [35].

Table 3. Effect of probiotics on AFM1 concentration.

Fermentation	No.	Before	After
Min	10	0.075*	0.068
Max	10	0.207*	0.198
Mean ± SE	10	0.125 ± 0.0165	0.117 ± 0.0164

Means carrying superscripts are significantly different at $p = 0.014$.

Conclusion

The presence of AFM1 in 49% of examined raw milk samples indicate widespread occurrence of AFM1 in market milk in Aswan province, Egypt which considered possible hazards for consumers, while the absence of AFM1 from UHT milk indicates that the type of milk is safer. So, regular monitoring of AFM1 in market milk is necessary for evaluating their contamination status. Mixed starter culture of *S. thermophilus* and *L. bulgaricus* could be used as a biological agent for the reduction of AFM1 in milk.

Conflict of Interests

None of the authors have any conflict of interest to declare.

Authors' contribution

All listed authors have made substantial contributions to the research design, the acquisition, analysis, or interpretation of data; and to drafting the manuscript or revising it critically; and that all authors have approved the submitted version.

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