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DETECTION OF AFLATOXIN M_1 IN MILK AND SOME DAIRY PRODUCTS IN DAMIETTA GOVERNORATE, EGYPT

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ABSTRACT

The mycological quality of cow's raw milk and cheese in Egypt has been extensively studied. However, rare data was found on the content of AFM_1 in them. Thirty random samples of dairy products that were purchased from different regions in Damietta governorate (July to September 2010) were analyzed for AFM_1 by HPLC and screened for the presence of fungi. Total count of fungi in milk ranged from 8 to 37 colony/ml, the raw milk samples were found to be contaminated with eight species of fungi belong to two genera; Aspergillus and Mucor. The Aspergillus group was the most prevalent fungi in all examined samples, within these species, A. niger, A. fumigatus, A. flavus, A. ochraceus, A. terreus, A. parasiticus, A. restirictus and Mucor species. White cheese samples were contaminated with five fungal genera, A. fumigatus, A. japonicus saito, A. ochraceus, A. terreus, A. flavus, Penicillium nigricans, Trichoderma , Rhizopus, Cochliobolus lunatus and Cochliobolus spicifer and the total fungal count ranged from 7 to 38 colony/g white cheese sample. On the other hand, hard cheese samples were contaminated with ten fungal species belong to four genera, A. fumigatus, A. flavus, A. parasiticus and A. terreus, A. japonicus saito, Cladosporium, A. niger, A. ochraceus, Mucor sp. and Candida spp. and the total fungal count ranged from 12 to 65 colony/g. AFM_1 was detected in milk, white and hard cheese and its concentrations ranged from 0.06 to 2.06, 0.26 and 0.58 to 2.9 ppb, respectively. AFM_1 levels in 15 out of the 30 samples were found to be higher than the maximum tolerable limit set by the Egyptian Ministry of Health. This study concluded that the AFM_1 level in the cow's milk, white and hard cheeses in the collected samples were higher than permissible levels and constitutes health hazard to consumers in these areas.

Key words: *Aflatoxin M_1, HPLC, Milk, Cheese, Fungi.*

INTRODUCTION

Milk and its products are the major nutrient for human especially children due to its high nutritional value. It is high in protein and a valuable source of calcium, vitamins and antioxidants (Zeluta *et al.,* 2009). These products may be contaminated with aflatoxin residues which extensively threaten the human health. (Michalski and Januel, 2006). Aflatoxins (AFs) are typically found as secondary metabolites of *Aspergillus flavus and Aspergillus parasiticus* and to a lesser extent *A. nominus* and other fungi (Migahed *et al.,* 2003). Epidemiological studies have shown that prolonged exposure to AFB_1 can cause liver cancer, especially in persons with hepati-

tis B antigens (Stubblefield *et al.,* 1983). Consequently, the World Health Organization (WHO) classifies AFB_1 and its metabolites as a human carcinogens (Anklam *et al.,* 2002) .

Aflatoxin M_1 (AFM_1) is a hydroxylated metabolite of AFB_1 and can be detected in cow's milk and other dairy products made from it specially cows that have ingested feed contaminated with AFB_1 (IARC, 1993; Zinedine *et al.,* 2007). Because AFM_1 is resistant to thermal inactivation (pasteurization and autoclaving) storage of various dairy products was not effective in the reduction of this toxin (Park, 2002). Therefore, many countries have set regulations to control the levels of AFB_1 in foods. The maximum level of AFM_1 in liquid and dried milk or processed milk products should not exceed 50 ng/kg (CAC, 2001; Rastogi *et al.,* 2004 and Aycicek *et al.,* 2005). However, according to US regulations, the level of AFM_1 in milk should not be higher than 50 ng/kg (Stoloff *et al.,*1991). In Egypt, the fluid milk and dairy products should be free from AFM_1 (Egyptian Regulations, 1990). In spite of the regulatory control measures taken by many countries, production of aflatoxin-free milk is not always achieved (Galvano *et al.,* 1996).

Although AFM_1 is less carcinogenic than AFB1 (2-10% of potency), it is also a health danger. It has comparable liver toxicity, can reduce the immunity of infants, and is considered to be a possible human carcinogen (2B) by the International Agency for Research on Cancer (IARC) (Sun and Chen, 2003).

Damietta governorate is one of the famous governorates producing milk and dairy products. These materials can be subjected to microbial food spoilage and particular fungal contamination. Therefore, the aim of this study was to analyze the composition and diversity of the fungal flora of raw milk and cheese and to investigate the presence of AFM_1 in different cow's milk samples and other dairy products at Damietta governorate.

MATERIALS AND METHODS

Sampling

A total of thirty samples (10 of each product) of local fluid cow's raw milk, enveloped fresh old white cheese and enveloped fresh old hard cheese were collected from five regions (EL Zarka, Farskour, Kafr Saad, Damietta City and New Damietta City) in Damietta Governorate. All samples were randomly purchased during the period of July to September 2010 from supermarkets. Raw milk samples were collected directly from bulk tanks using sterile 1L bottles. The bottles were placed on ice and were sent to the laboratory as quickly as possible where they were stored at -4°C until analysis. The samples were analyzed within 24 h- 48 h.

Methods

Isolation and Identification of Fungi

Fungal species were isolated from cow's milk, white and hard cheese samples according to Harrigan and Margaret (1966). One mL of cow's milk, one gm of white and hard cheese samples were mixed with sterilized distilled water to a final volume of 10 ml. One ml of the diluted sample was spread on to Czapek's agar or potato dextrose agar (PDA) media and incubated at 28-30 °C for 3-5 days. Subculturing was repeated successively until

pure fungi were obtained and were identified according to their cultural morphology and spores description (Raper *et al.,* 1968, Domsch *et al.,* 1980 a and b).

Extraction of AFM_1

AFM_1 was extracted according to method of Association of Official Analytical Chemists (AOAC 986.16, 2007). In brief, a 20 ml of raw milk was mixed with 20 ml of warm water and 10 ml of H_2O-CH_3CN wash solution and 150 µl of CH_3CN were added to it. This mixture was added to the washed silica gel column and eluted with ether at 10 ml/min flow rate. Then AFM_1 was eluted with 7 ml of CH_2CL_2-alcohol at the same flow rate. The eluant was collected in a clean tube, evaporated to dryness under nitrogen steam. The dried residue was dissolved in CH_2CL_2, evaporated to dryness as above and kept for derivatives preparation.

For cheeses samples, ten grams of cheese were mixed with one hundred ml distilled water in a blender for twenty minute, then filtered and 20 ml of filtrate were transferred in capped tube. Centrifuged at (4000 rpm for 15 min) and the supernatant removed for extraction. The extraction is carried out as described above.

Determination of AFM1 by HPLC

200 µl hexane and 200 µl of trifluoracetic acid were added to dry residue of each sample and standard vortexed for 30 sec. Kept for 10 min at 40°C in water bath, then evaporated to dryness under nitrogen. 2 ml (water- acetonitrile, 75:25 v/v) was added to each vial to dissolve residue and vortexed and used for HPLC analysis.

HPLC conditions: The mobile phase consists of water-isopropanol- acetonitrile (80:12:8). The separation was performed at ambient temperature at a flow rate of 1.0 ml/ min. The injected volume was 20 µl for either the standard solutions or the sample extracts. The fluorescence detector was operated at an excitation wavelength of 365 nm and an emission wavelength of 435 nm. AFM_1 concentration in samples was determined from the standard curve, using peak area for quantitation (AOAC, 2000).

RESULTS AND DISCUSSION

Fungal species in cow's milk and cheeses samples

The results indicated that milk samples were contaminated with eight species of fungi belong to two genera, *Aspergillus* group was the most prevalent fungi in all examined samples. Where, *A. niger* and *A. fumigatus* constituted 26 % of the total fungal isolates, followed by A. *flavus* (19 %), *A. ochraceus* (13%). *A. terreus* (6%). While each of A. *Parasiticus, A. restirictus* and Mucor sp. represented the lowest occurrence of 3.0%. Also it was obvious that the cow's milk samples collected from El-Zarka and Damietta city recorded the higher total fungal count (TFC = 37.0 colony/ ml), followed by Kafr Saad (21.0 colony/ ml), Farskour (19.0 colony/ ml) and New Damietta City (8.0 colony / ml) (Fig. 1).

In white cheese samples, it was found that *A. niger* was the predominant and constituted (30 %) of the total fungal count of the isolates, followed by *A. fumigatus* and *A. japonicus saito* (15 %) for each one. Whereas *A. ochraceus* (12%). *A. terreus* (8%). Each of *A. flavus,*

Penicillium nigricans, Trichoderma, Rhizopus, Cochliobolus lunatus and *Cochliobolus spicifer* constituted (4.0%). In addition, the white cheese samples collected from El-Zarka, Farskour and Damietta recorded the higher total fungal count (TFC = 38.0, 27and 26 colony / g), respectively. Followed by Kafr saad and New Damietta (10.0 and 7.0 colony /g), respectively, (Fig. 2).

Results of analysis of hard cheese samples showed the occurrences of *A. fumigatus, A. flavus, A. parasiticus and A. terreus* were 34%, 19%, 13% and 9%, respectively. *Aspergillus japonicus saito* and *Cladosporium* comprised 6% for each one . On the other hand, the frequency of each of *A. niger, A. ochraceus, Mucor* and *Candida spp.* was 3.0%. From the current results, it was clear that, the hard cheese samples collected from El-Zarka, New Damietta and Farskour recorded the higher total fungal count (65.0, 37.0 and 26.0 colony /g), respectively. On the other hand, Kafr saad and New Damietta represented the lower occurrence (15.0 and 12.0 colony /g), respectively, (Fig. 2).

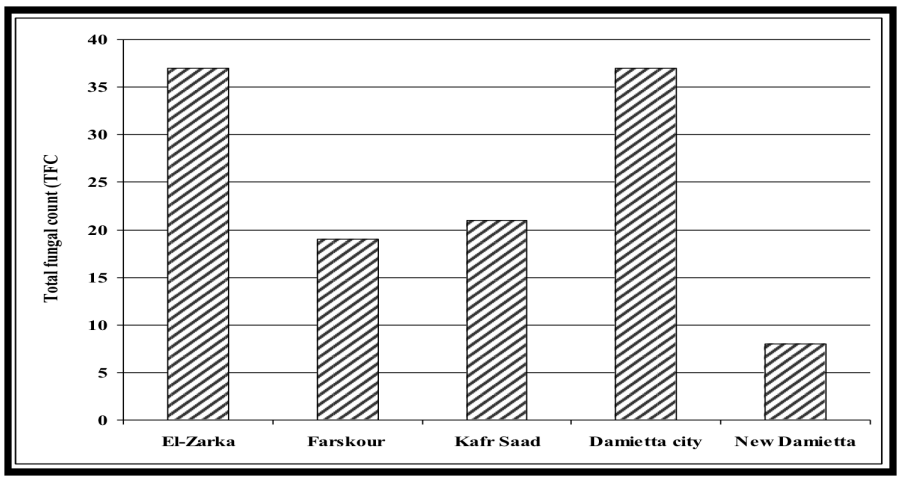

Fig. (1) : TFC associated with cow's milk samples collected from different regions in Damietta governorate.

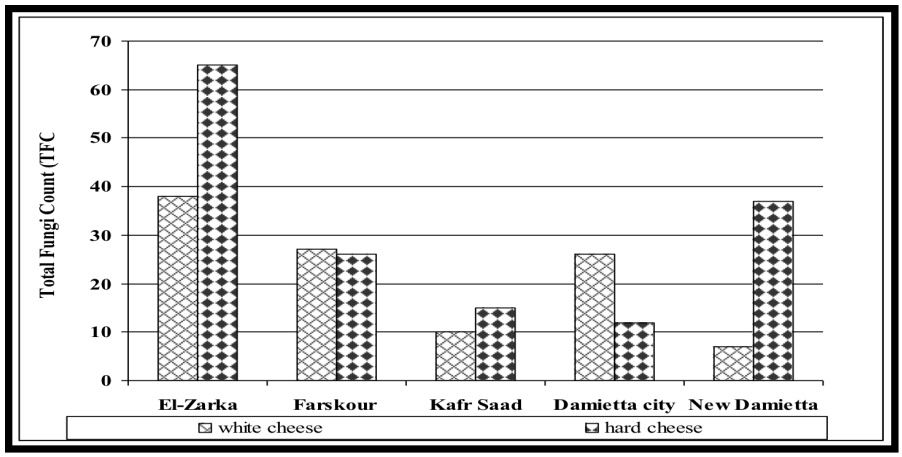

Fig. (2) : TFC associated with white and hard cheese samples collected from different regions in Damietta governorate.

Our results are in agreement with those of Kurtzman *et al.,* 1987 who isolated *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius* from chesses. Also in agreement with Taniwaki *et al.,* 2001 who concluded that commonly isolated fungi from cheese *include Penicillium, Aspergillus, Cladosporium, Geotrichum, Mucor* and *Trichoderma*, also reported that cheese is the only product really susceptible to fungal growth. Development of moulds on the surface of cheeses is thought to be the sign of spoilage. However, certain mould species that have low toxigenic capacity are used to produce special products that have different organoleptic characteristics such as mould ripened cheeses. These types of cheeses are ripened by species of *Penicillium*, which grow within the cheese or by the growth of surface moulds and they are produced by using starter mould culture or produced spontaneously (Beresford *et al.,* 2001).

Hocking and Faedo, (1992) had explained the growth of fungi on cheeses such as (*Aspergillus fumigatus, Fusarium oxysporum,* and *Penicillium expansum*) to their ability to develop at lower oxygen levels, such as the centre of cheeses or in hermetically sealed products. Other species, *Aspergillus flavus* are xerophilic, it can grow in low water activity substrates<0.80 values, others are salt tolerant. Therefore a quick salting of the cheeses is detrimental to other moulds.

Detecion of Aflatoxin M_1 (AFM_1)

I. In cow's milk

All cow's milk samples collected from EL Zarka region, New Damietta, Farskour, Damietta City and kafr Saad regions are contaminated with AFM_1 at higher levels of 0.58, 0.53, 2.06, 0.58 and 0.52 ng/ml, respectively, (Fig. 4 a, b & c). Regulatory limits throughout the world are influenced by economic considerations and may vary from one country to another (Stoloff *et al.,* 1991; Van Egmond, 1989). The European Community and Codex Alimentarius has prescribed that the maximum limit of AFM_1 in liquid, dried or processed cow's milk products can not exceeds 0.05ng/g (Codex Alimentarius Commission (CAC 2001) and European Commission Regulation, EC 2001).

AFM_1 concentrations in our samples were higher than the permissible limits set by international organizations, so we must addressed this problem with all seriousness. These results agreed with Matteo *et al.* (2006) and Alborzi *et al.* (2006) whom reported that 17.8% of the samples had AFM_1 greater than the maximum tolerance limit (50 ng/l) accepted by European Union. Also agreed with Zinedine *et al.* (2007) as the mean value of AFM1 was 18.6 ng/ L and with Ghanem and Orfi (2009), and with Okeke *et al.* (2012). El Khoury *et al.* (2011) who found that AFM_1 in 40.62% of examined cow's milk samples, but in disagreement with Mohamadi and Alizadeh (2010) as they found that all cow's milk samples analyzed showed AFM_1 concentrations lower than the permissible level of 50 ng/ml.

According to Stoloff (1980), milk has the greatest potential for introducing AFs residues from edible animal tissues into the human diet, and taking into account that pasteurization processes and even those using ultra high temperature UHT techniques do not affect AFM_1 concentration because of its

heat stability Galvano *et al.* (1996). The major metabolite of AFB_1 is aflatoxin M_1 (AFM_1), which is detectable in the urine, blood, cow's milk, and internal organs of animals ingesting AFB_1-containing feed, ranges from 0.5 - 5% (Carvajal *et al.,* 2003). Generally, cows could excrete milk with up to 0.05 µg/L of AFM_1 if their daily intake of AFB_1 reaches 70 µg (Hussein and Brasel, 2001). Because milk is the main nutrient for growing young eater, whose vulnerability is noteworthy and potentially more sensitive than that of adults, the occurrence of AFM_1 in human breast milk, commercially available milk, and milk products represents one of the most serious problems of food hygiene. For this reason, many countries have regulations to control the levels of AFB_1 in feeds and to propose the maximum permissible levels of AFM_1 in milk to reduce this risk (Rastogi *et al .,* 2004).

II. In white cheese and hard cheeses.

AFM_1 in white cheese was detected in only sample of kafr saad with a level of 0.26 ng/g. All hard cheese samples collected from Damietta city, Kafer saad, Farskour and El-Zarka regions are contaminated with AFM_1 at higher levels of (2.57, 2.9), 2.21, 1.46 and 1.1 ng/g, respectively, (Fig. 3). and other dairy products are always at risk of being contaminated with AFM_1. Parallel to the increasing amount of cow's milk and dairy product consumption, studies on the presence of AFM_1 in cheese products have been increasing globally as well as in Egypt.

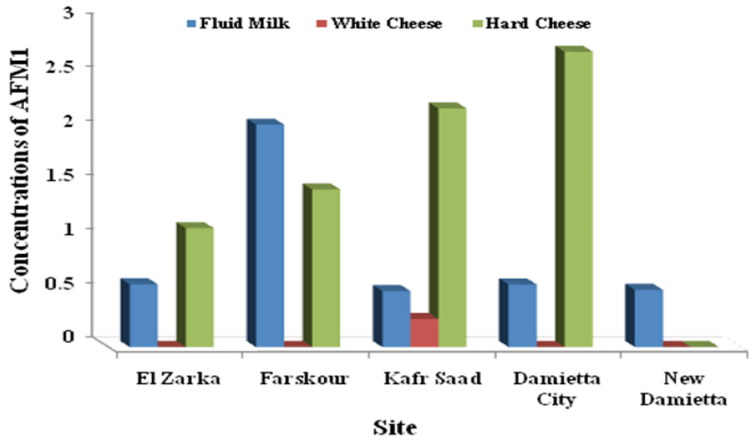

Fig. (3) : Mean concentrations of AFM1 in fluid cow's milk, white cheese and hard cheeses from different regions in Damietta governorate.

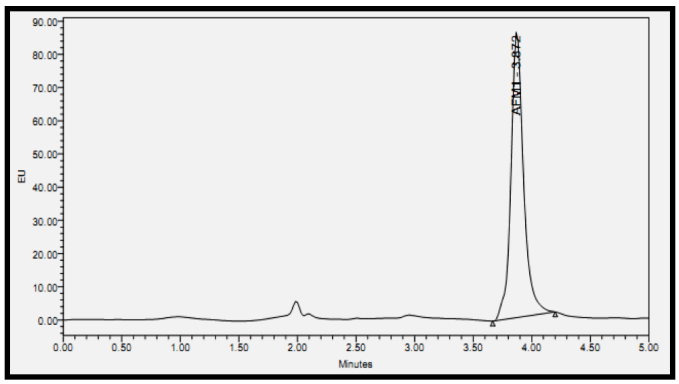

Fig. (4a) : HPLC chromatogram standard of AFM_1.

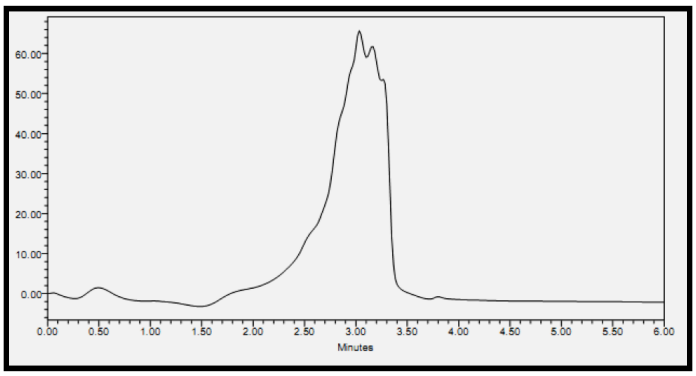

Fig. (4b) : HPLC chromatogram of negative sample.

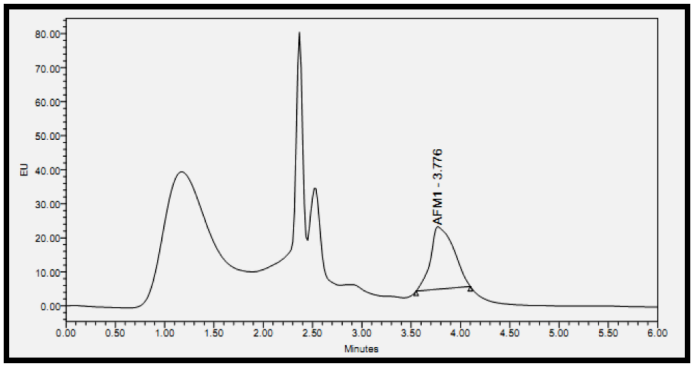

Fig. (4c) : HPLC chromatogram of AFM_1 in positive sample of hard cheese from Kafr Saad.

Occurrence of AFM_1 in cheese can be attributed to three possible causes; AFM_1 present in raw milk, or synthesis of AFs (B_1, B_2, G_1 and G_2) by *Aspergillus flavus* and *Aspergillus parasiticus* growing on cheese (Zerfiridis, 1985), and occurrence of these toxins in dried milk used to enrich the milk which is being used in the production of cheese (Blanco *et al.,* 1988). However, the increase in AFM_1 concentration in cheese has been explained by the affinity of AFM_1 for casein (Grant and Carlson, 1971; Applebaum *et al.,* 1982). The high levels of AFM_1 in milk and cheese samples implies the presence of very high AFB_1 levels in feed itself. The practice of farmers of Damietta governorate in harvesting hay in the summer and feed it to the cattle during the winter. Fungi growing in haystacks may easily produce toxins in inappropriate storage conditions. Following the consumption of highly contaminated feed with AFB_1, conversion of AFB_1 to AFM_1 takes place in the liver and leads to elevated levels of AFM_1 in the milk. Therefore, it is important to reduce the occurrence of AFB_1 in feedstuff and take prophylactic measures to prevent factors enhancing toxin production such as temperature, humidity, and moisture content of the feed as well as pH and mechanical damage to the grain affecting mold production. Hence, management practices in harvest and storage regarding the aforementioned factors could decrease AFs occurrence in feed. These results suggest that it is important to prevent toxin production in these products from the production stage to consumption as well as creating effective detoxification processes (Personal communication).

The results obtained show that all concentrations were present in these samples higher than internationally permissible limits. These results are in agreement with those of Hasan *et al.* (2005) who reported that AFM_1 levels in 19 (8.52%) of 223 dairy product samples were higher than maximum tolerable limit of the Turkish Food Codex. Also are in agreement with Dashti *et al.* (2009) who found that one sample being above the regulatory limit (0.250 µg/ kg). Also agreed with Amer and Ekbal Ibrahim, (2010) they revealed that all positive samples of raw milk and cheeses are exceeding Egyptian regulations in addition to, Ebrahim (2012) who mentioned that concentrations of AFM_1 in eleven samples of cheese samples (13.8%) and three samples of yoghurt samples (5.0%) were higher that maximum tolerance limit accepted by European Union/Codex Alimentarius (250 ng/L). While, our results differ with Martinsl *et al.* (2007) as they found that eight samples (6.25%) contained levels of AFM_1 at the maximum permissible level (0.05 µg/ kg). Also in disagreement with Mohamadi and Alizadeh, (2010) who found that all milk samples analyzed showed mean AFM_1 concentrations lower than the permissible level of 50 ppb (23.22±8.65, 19.53±7.47 ppb in pasteurized milk, and UHT milk, respectively). The mean levels of AFM_1 contamination were 43.31±18.51 ppb in Feta cheeses and 21.96±3.23 ppb in creamy cheeses.

CONCLUSION

In conclusion, the concentrations of AFM_1 in cow's milk and dairy products samples were higher than international permissible

limits which constitutes a real problem that must be addressed with all seriousness. The authors strongly recommend that AFM_1 and/or other mycotoxins should be routinely monitored, frequent analytical surveillance by Food Control Agencies to reduce the incidence of mycotoxin contamination in milk and dairy products in Egypt .

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الملخص العربى

الكشف عن الأفلاتوكسين إم١ فى اللبن وبعض منتجات الألبان فى مناطق مختلفة بمحافظة دمياط، مصر

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قد يتواجد الافلاتوكسن إم١ في الألبان ومنتجاته نتيجة تغذية الأبقار على أعلاف ملوثة بالافلاتوكسين ب١ وحيث أن نادرا ما تتعرض الدراسات لرصد تركيز الأفلاتوكسين إم١ في اللبن والجبن بمحافظة دمياط، لذا تهدف هذه الدراسة لتقدير مستويات الافلاتوكسن إم١ في الألبان والجبن الأبيض والجبن الرومى الشائع استخدامها في محافظة دمياط. تم تجميع عدد ٣٠ عينة من الألبان ومنتجاته عشوائيا من مناطق مختلفة بالمحافظة وهى الزرقا، فارسكور، كفر سعد، دمياط القديمة و دمياط الجديدة خلال الفترة من (يوليو ٢٠١٠ إلى يوليو ٢٠١١) وقد تم تحليل العينات باستخدام جهاز الكروماتوجرافى عالى الكفاءة بالإضافة إلى فحص وجود الفطريات والافلاتوكسينات بالعينات. وقد أظهرت النتائج أن عينات الألبان ملوثة باثنين من الأجناس وثمانية من الأنواع حيث كان لفطر الأسبرجلس السيادة في الظهور في كل عينات الدراسة، حيث تراوح العد الفطري الكلى لعينات الألبان مابين ٨ و ٣٧ مستعمره/مل لبن، بينما تبين أن عينات الجبن الأبيض ملوثة بخمس أجناس وإحدى عشر نوعا وقد تراوح العد الفطري الكلى لها مابين ٧ و ٣٨ مستعمره/جم جبن ابيض). على الجانب الأخر، أظهرت النتائج أن عينات الجبن الرومى ملوثه بأربع أجناس وعشره من الأنواع كما تراوح العد الفطري لها مابين ١٢ و ٦٥ مستعمره/جم جبن راس. أيضا تم تعيين تركيز الأفلاتوكسين إم١ في الألبان وقد تراوح التركيز مابين و ٢٠,٠٦ جزء في البليون. بينما بلغ تركيز افلاتوكسين إم١ في عينات الجبن الأبيض إلى ٠,٢٦ جزء في البليون في حين تراوح تركيز الأفلاتوكسين إم١ في عينات الجبن الرومي مابين ٠,٥٨ و ٢,٩ جزء في البليون. وقد أثبتت النتائج أن تركيز الأفلاتوكسين إم١ في عدد ١٥ عينه من إجمالي ٣٠ عينة أعلى من الحدود المسموح بها في مصر والتي تنص على أن الألبان ومنتجاته من الجبن الأبيض والرومى يجب أن تكون خالية من الأفلاتوكسين إم١ ونستنتج من الدراسة أن لبن الأبقار الموجود بمحافظة دمياط ملوث بالافلاتوكسين إم١ بتركيزات تمثل خطرا على صحة الإنسان.

JOESE 5

DETECTION OF AFLATOXIN M_1 IN MILK AND SOME DAIRY PRODUCTS IN DAMIETTA GOVERNORATE, EGYPT

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