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Short Communication

Determination of Aflatoxin M1 and Ochratoxin A in Milk and Dairy Products in Supermarkets Located in Mansoura City, Egypt

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Abstract | In this study, the occurrence of different mould species from milk samples, milk powder, roomy cheese and kariesh cheese samples in Mansoura city, Egypt was determined. Out of 200 randomly screened samples, 67(33.5%) were confirmed positive for the presence of different mould species. The overall incidence rate in raw milk, milk powder, roomy cheese and kariesh cheese were 20%, 38%, 48% and 28% respectively with a mean mould counts, 2.284 ± 0.488 × 10^2 cfu/mL, 1.416 ± 0.155 × 10^2 cfu/g, 2.727 ± 0.770×10^2 cfu/g and 1.950 ± 0.578 × 10^2 cfu/gmL respectively. Significant variation in the incidence of different mould species was detected among different examined samples. Various mould species were recovered from these examined samples including, *Aspergillus spp., Penicillium spp., Mucor spp., Geotrichum spp., Cladosporium spp., Byssochlamys spp., Nigrospora spp., Rhizopus spp., Acremonium spp., Fusarium spp. and Paecilomyces spp.* Aflatoxin M1 (AFM1) and Ochratoxin A (OTA) were estimated in a total of 40 raw milk and dairy products samples by immunoaffinity column (IAC) and was measured by VICAM fluorometer. AFM1 was detected in 70% of all examined samples, while; OTA was detected in %80 of all examined samples. A significant difference between concentrations of OTA (p=0.037) and concentrations of AFM1 (p=0.001) among different milk products were detected. All positive samples harboring AFM1 exceeded the Egyptian regulation for AFM1 in milk. In conclusion, Presence of moulds and mycotoxins in milk and different dairy products with high percentage indicates public health hazards that require strict hygienic conditions in dairy products industry.

Keywords | Moulds, Dairy products, Mycotoxins, Aflatoxin M1, Ochratoxin A

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Milk and its products are being nutritious food for human and they provide a favorable environment for the growth of various microorganisms. Yeast and moulds can grow in milk and different dairy products at suitable conditions of temperature and moisture (Barrois et al., 1997). Mycotoxins are secondary metabolites of low molecular weight produced by naturally occurring fungi in different kinds of foods and feed stuffs, particularly cereals, peanut, meat, meat products, milk, milk products or eggs (Brown et al., 2001). Mycotoxins can be found in dairy products from two origins, indirect contamination, which results when dairy cows ingest feed that contains mycotoxins which pass into the milk such as aflatoxin M_1 and direct contamination, which results from accidental growth of moulds secreting aflatoxins. (Sengun et al., 2008). Fungal metabolites have toxic effects when present in foods, their effects range from acute (for example, liver or kidney deterioration), to chronic (for example, liver cancer), mutagenic, and teratogenic; and resulting symptoms range from skin irritation to immunosuppression, birth defects, neurotoxicity or death (ICMSF, 1996). Mycotoxins have many economic impacts including reduced livestock production, increased health care and veterinary care costs, disposal of contaminated foods and feeds, and investment in research and applications to reduce severity of the mycotoxin problem (Zain, 2011). There are many mycotoxins of worldwide public health importance including, Aflatoxins, ochratoxins (OAs), fumonisins, zearalenone

(ZEN), and trichothecenes (FAO, 2001). Aflatoxins are a group of toxic compounds produced by certain strains of Aspergillus flavus, Aspergillus parasiticus and rarely Aspergillus nomius (Van Egmond, 1989; Creppy, 2002). The major aflatoxins are called B1, B2, G1, and G2 (based on their fluorescence under UV light (blue or green) M1 and M2 (produced in milk and dairy products) (D'Mello and MacDonald, 1997). Aflatoxin M1 (AFM1) may be found in the milk of animals that are fed on aflatoxin B1 (AFB1) containing feed (Van Egmond, 1991). Ochratoxin A (OTA) is a mycotoxin produced by different species of Aspergillus (A. ochraceus, A. melleus, A. sulphureus, A. Niger, A. carbonarius, A. awamori) and Penicillium (P. verrucosum, P. crysogenum and P. nordicum) (Bayman and Baker, 2006; Magan, 2006; Zheng et al., 2005). Ochratoxin A has hepatotoxic, nephrotoxic and teratogenic effects (Boudra and Morgavi, 2006). OTA has been classified by the International Agency of Research in Cancer (IARC) as a carcinogenetic of 2B class (Muscarella et al., 2004). Mycotoxin analysis in food and feed is generally a multi-step process comprised of sampling, sample preparation, toxin extraction from the matrix (usually with mixtures of water and polar organic solvents), extract clean up and finally detection and quantitative determination (Koppen et al., 2010). In Egypt, the data concerning mycotoxins contamination in foods, especially aflatoxin M1 and ochratoxin A from dairy products, are lacking and these collected data are the first step for prevention and control of mycotoxins hazard. This study, therefore, was conducted to determine the prevalence of different mould species in milk and dairy products from different supermarkets located in Mansoura city, Egypt, with estimation of mycotoxins in the examined samples including, AFM1, OTA and to compare their levels with the permissible limits if founded.

A total of 200 samples of raw milk, milk powder, roomy cheese and kariesh cheese (50 each) were collected randomly from different supermarkets located in Mansoura city, Egypt. The collected samples were transferred directly to the laboratory with a minimum of delay under a septic condition. Each sample was divided into two parts. The first part was prepared for mycological examination and the second one was kept frozen for mycotoxins estimation. Preparation of samples for mycological examination was done according to (APHA, 1992).

For Fungal isolation and identification, 100 µL from each sample were spread onto plate of Sabouraud dextrose agar (SDA) medium by using a bent sterile glass spreader rod (hockey stick). The inoculated plates were incubated at 25°C for 5-7 days and examined daily (Pitt et al., 1992). Mould count in each plate was recorded after 48 hours of incubation. Moulds identification was done according to Pitt and Hocking (1997).

AFM1 estimation in milk and dairy products samples including, samples preparation, toxin extraction, clean up via IAC, toxin elution and measure with Vicam flourometry were done following the Vicam AFM1-FL+\4.2 -GN-MC9954-4**.** 40 mL of raw milk samples were centrifuged at 2000 X g for 10 min, bottom layer (skimmed part) was carefully collected for analysis. 10g of milk powder samples were reconstituted with 100 mL of preheated purified water gradually until a homogeneous mixture was obtained; 40 mL of reconstituted milk was used as raw milk samples. Concerning to the kariesh cheese samples, 10 gm of samples were homogenized with 100 mL distilled water then 40 mL of homogenized sample was used as raw milk samples. Preparation of roomy cheese was done according to (AOAC 991.31, 1991), 25 gm of roomy cheese samples were mixed with 5g of Nacl and blended with 125 mL methanol:water (70:30) at high speed for 2 minutes . The extract was filtered through a fluted filter paper. 15 mL of the filtered extract was diluted with 30 mL distilled water, then filtered through 1.5µm glass microfibre filter. 15 mL of the filtered extract (15 mL = 1.0 g sample equivalent) was used for analysis. The final extract of each sample was passed through IAC (Vicam Afla M1 FL+ Column, Vicam, Watertown, MA, USA) completely at a rate of 1-2 drops/second until air came through column. Column was washed twice using 10 mL of washing solution. Toxin was eluted using 1 mL methanol (HPLC grade) a rate of 1drop/second until air came through column and all of the sample was eluated (1.0 mL) and collected in a glass cuvette. One mL diluted AflaTest Developer was added to the elute in the cuvette and mixed well, then placed in a calibrated fluorometer (VICAM Series 4,4EX, MA, USA).

For Ochratoxin A estimation in raw milk and milk products**,** Milk samples were prepared according to Iha et al. (2014). 10g of each milk powder sample was reconstituted with 100 mL of preheated purified water gradually until a homogeneous mixture was obtained and then used as raw milk samples. Preparation of cheese samples according to Scott (2002), 25 gm of sample was mixed with extraction solvent (methanol) and aqueous sodium bicarbonate followed by high speed blending. The extract was filtered through fluted filter paper. The diluted extract was filtered through 1.5µm glass microfibre filter then directly passed through IAC (Vicam Ochratest affinity column, Vicam, watertown, MA, USA) according to

Data were analysed by using SPSS, 2004 (version 16.0). Chi square test was used to test association between incidences of different mould count in milk products, also analysis of difference in a concentration of different toxin in milk products at p-value <0.05.

A total of 200 samples including raw milk, milk powder,

roomy cheese and kariesh cheese (50 each) were evaluated mycologically, Moulds were detected in 67(33.5%) of the examined samples. Mould incidence rates in raw milk, milk powder, roomy cheese and karish cheese were 10 (20%), 19 (38%), 24 (48%) and 14(28%), respectively (Table 1). Statistical analysis showed significant difference between different examined samples in the prevalence of mould species (p value=0.0191). Roomy cheese samples showed the highest prevalence of moulds (48%) and the highest mean mould count (2.727±0.770 $\times 10^2$ cfu/g), the source of this high contamination of cheese may be raw materials as milk, starter culture and brine, air, water, equipments, workers walls and shelves of ripening room, etc. (Chapman and Sharpe, 1990).

Table 1: The mean mould counts in different examined samples

Samples	Examined samples	Positive samples		Mould count cfu/g or mL		
		No.	%	Min	Max	Mean ±SE
Raw milk	50	10	20	1×10^2	6×10^2	2.28±0.49
Milk powder	50	19	38	1×10^2	2×10^2	1.42±0.16
Roomy cheese	50	24	48	1×10^2	8×10^2	2.73±0.77
Kariesh cheese	50	14	28	1×10^2	6×10^2	1.95±0.58
Total	200	67	33.5	1×10^2	8×10^2	2.08±0.29

Aspergillus niger, *A. flavus*, *A.terreus*, *A.versicolor* and *A. oryzae* were isolated from the examined raw milk samples, *A.niger* was the predominant species. Statistical analysis showed a high significant difference in the prevalence of mould species in the examined raw milk samples (p=0.0026). Wide distribution of mould spores in the unsanitized environment considers the main cause for milk contamination during milk production, transportation and/or storage (Bullerman, 1979; Samson et al.,1988). Mould count in the examined raw milk samples was ranged from 1×10^2 to 6×10^2 cfu/mL with a mean ± SE value of 2.284 ± 0.488 $\times 10^2$ cfu/mL (Table 1). These results are higher than (Desmasures et al., 1997) and (Bille et al., 2009) and lower than (Tasci, 2011).

Concerning to milk powder samples, out of 50 examined samples, 19(38%) samples were contaminated with different mould species including, *A.flavus, A.niger, P.chrysogenum, P. funiculosum and P.corylophilum*, *Paecilomyces spp., Cladosporium spp., Nigrospora spp.* and *Rhizopus spp*. Statistical analysis showed no significant difference in the prevalence of mould species in the examined milk powder samples. Presence of moulds in milk powder may be resulted from contaminated air, bad environment in some factories as dispersed milk powder on ground, equipments, workers' clothes which promote growth of micro-organisms, uncontrolled sterilization, reinfection after pasteurization (Bonfoth, 2004) or use of low quality milk, utensils during stage of production, processing, transportion or storage. (Cross, 1997). Mould count in the examined milk powder samples was ranged from 1×10^2 to 2×10^2 cfu/g with a mean ± SE value of 1.416±0.155 $\times 10^2$ cfu/g. this count exceeded the Egyptian permissibile limit which should not exceed 10 cfu/g (Egyptian standards, 1648/2005) which has a high risk on human health.

Out of 50 hard (roomy) cheese samples, (24) 48% were contaminated with different species of moulds (Table 1). *A.parasiticus A.niger A.terreus*, *A.fumigatus*, *P.oxalicum*, *P. cyclopium*, *Cladosporium spp., Byssochlamys spp. and Geotrichum spp.* were detected in the examined samples; *A.niger* was the predominant species (Table 2). Statistical analysis showed a high significant difference in the incidence of mould species in the examined roomy cheese samples (p=0.0005). These results are nearly similar to Sabreen and Zaky (2001) and EL-fadaly et al. (2015). Mould count in the examined roomy cheese samples ranged from 1×10^2 to 8×10^2 cfu/g with a mean ± SE value of 2.727±0.770 $\times 10^2$ cfu/g. which exceeded the Egyptian permissible limit for presence of moulds in hard cheese according to the Egyptian standards 1007/2005 (not exceed 10 cfu/g).

In this study, a total of 50 soft (kariesh) cheese samples were evaluated for the presence of mould species, Different mould species were isolated including *Geotrichum spp.*, *A.flavus*, *A.niger, Mucor spp., Fusarium spp.* and *Acremonium spp.*. *Geotrichum spp.* was the predominant species in the positive samples (Table 2). Statistical analysis showed that a high significant difference was detected in the incidence of mould species in the examined kariesh cheese samples (p=0.002). These results are nearly similar to El-Diasty and Salem (2008) and with Sabreen and Zaky (2001). Mould count in the examined kariesh cheese samples was ranged from 1×10^2 to 6×10^2 cfu/g with a mean ± SE value of 1.95 ± 0.578 $\times 10^2$ cfu/g which exceeded the Egyptian permissible limit (not exceed 10 cfu/g) according to the Egyptian standards 1008/2005 .Mould contamination may represent a great hazard even in small counts if the package is opened and kept under uncontrolled temperature and moisture conditions which activate mould to produce toxins (El-Shazly, 2002) so the viable counts of moulds are not a reliable indicator of mycotoxin production (Pitt, 1984).

Presence of moulds as Aspergillus or Penicillium genera didn't indicate presence of their mycotoxins in dairy products due to some strains are non-toxigenic or even toxigenic strains didn't produce their toxins (Zerfiridis, 1985). While presence of mycotoxin in foods and feed depend on environmental conditions related to storage and other extrinsic factors as climate or intrinsic factors such as fungal

Table 2: Mould genera recovered from the examined samples

Moulds	Positive Samples							
	Raw milk		Milk powder		Kariesh cheese		Roomy cheese	
	No.	%	No	%	No	%	No.	%
A.niger	7	63.63	4	21	1	6.66	10	33.33
A.flavus	1	9	2	10.52	1	6.66	-	-
A.parasiticus	-	-	-	-	-	-	7	23.33
A.terreus	1	9	-	-	-	-	4	13.33
A.oryzae	1	9	-	-	-	-	-	-
A.fumigatus	-	-	-	-	-	-	1	3.33
A.versicolor	1	9	-	-	-	-	-	-
Paecilomyces spp.	-	-	1	5.26	-	-	-	-
P.chrysogenum	-	-	3	15.78	-	-	-	-
P.funiculosum	-	-	2	10.52	-	-	-	-
P.corylophilum	-	-	1	5.26	-	-	-	-
P.oxalicum	-	-	-	-	-	-	1	3.33
P.cyclopium	-	-	-	-	-	-	1	3.33
Cladosporium spp.	-	-	4	21	-	-	2	6.66
Nigrosporia spp.	-	-	1	5.26	-	-	-	-
Rhizopus spp.	-	-	1	5.26	-	-	-	-
Byssochlamys spp.	-	-	-	-	-	-	3	10
Mucor spp.	-	-	-	-	3	20	-	-
Acremonium spp.	-	-	-	-	1	6.66	-	-
Fusarium spp.	-	-	-	-	1	6.66	-	-
Geotrichum spp.	-	-	-	-	8	53.33	1	3.33
Total	11	100	19	100	15	100	30	100

Table 3: AflatoxinM1 (AFM1) concentrations in examined samples

Samples types	Samples number	Positive		Range (ppb)		Mean of level ± SE
		No.	%	Min	Max	
Raw milk	10	6	60	0.013	0.125	0.061±0.015
Milk powder	10	8	80	0.013	0.021	0.016±0.001
Roomy cheese	10	8	80	0.01	0.021	0.015±0.001
Kariesh cheese	10	6	60	0.045	0.2	0.088±0.024
Total	40	28	70	0.01	0.2	0.041±0.008

strain specificity, strain variation and instability of toxigenic properties (Atanda et al., 2011).

A total of 40 samples of milk and dairy products (10 raw milk samples, 10 milk powder, 10 roomy cheese samples, 10 kariesh cheese samples) were examined for presence of AFM1. Aflatoxin M1 (AFM1) was detected in 60% of raw milk samples, ranging from 0.013 to 0.125 ppb with a mean ± SE value of 0.061 ± 0.015 ppb. Presence of AFM1 in the milk referred to feeding of animals on aflatoxin B1 (AFB1) containing feed which transformed in liver by hepatic microsomal cytochrome P450 to AFM1 and descend in milk (Frobish, et al., 1986). In addition to, a linear relationship between AFM1 in milk and AFB1 content of feed was reported (Van Egmond, 1989; Wood, 1991). The amount of AFM1 found in milk represents normally 1 to 2% of the ingested AFB1. However, it can be as high as 6% in high-producing cows (Veldman et al., 1992).

The Egyptian regulation (Egyptian regulation, 1990) for AFM1 in milk recommended that milk must be free from AFM1, in this study, all the positive samples exceeded the Egyptian regulation, while, EU regulation for AFM1 in milk (EU:European Commission (EC), 2006) recommended a maximum limit for presence of AFM1 in milk should not exceed 0.05 µg/kg, so in this study, 66.66% of the positive raw milk samples exceeded the EU regulation and 33.33% of these samples complied with it (Table 4). In comparison with FDA regulation (US Food and Drug Administration, 1996), which has a permissible limit of 0.5 ppb, 100% of our samples complied with its regulation for AFM1 in milk.

Table 4: AFM1 level in positive samples in relation to the permissible limits:

Positive Samples	Egyptian regulation,1990		EU: European Commission (EC), 2006	
	exceed	comply	Exceed	Comply
Raw milk	100%	0	66.66%	33.33%
Milk powder	100%	0	0	100%
Roomy cheese	100%	0	0	100%
Kariesh cheese	100%	0	50%	50%

Concerning to milk powder samples, 80% of all examined samples were contaminated by AFM1in a range of 0.013 to 0.021 ppb with a mean ± SE value of 0.016±0.001 ppb. Presence of AFM1 in milk power may refer to stability of AFM1 in heat treatment and drying as reported in most researches (JECFA, 2001). All the positive milk powder samples exceed the Egyptian regulation and comply with the EU regulation.

In kariesh cheese samples, 60% of the examined samples were contaminated with AFM1 in a range of 0.045- 0.2 ppb with a mean ±SE value 0.088 ± 0.024 ppb which exceeds the Egyptian regulation, while, 50% only of these samples exceeded the EU regulation. Concerning to roomy cheese samples, 80% of the examined samples were contaminated with AFM1 in a range of 0.01 to 0.021ppb with a mean ± SE value of 0.015±0.001 ppb. All the positive roomy cheese samples exceed the Egyptian regulation, while all of these samples comply with the EU regulation. AFM1may be found in the milk of animals that are fed with aflatoxin B1(AFB1) containing feed (Van Egmond, 1991) or dried milk used to enrich the milk used to make cheese (Blanco et al., 1988), in addition to the stability of aflatoxin M1 during ripening and AFM1 associated with casein, so that cheese curd contains a higher concentration than whey (Yousef and Marth, 1989). Significant differences were detected between concentrations of AFM1 toxin in different milk products (p=0.001).

OTA was detected also in milk and milk product samples and the results showed that (80%) of all examined samples were contaminated with OTA in a range of 0.34 to 13 ppb with a mean ± SE value of 5.134±1.822 ppb (Table 5). Presence of OTA in milk in spite of ruminal microflora and protozoa ability of its degradation may refer to change in diet (high concentrate proportion and high feeding levels) which lead to shift in microbial population and higher contamination potential (Mobashar et al., 2010; Özpinar et al., 1999).

In this study, OTA in raw milk samples ranged from 0.34 to 13 ppb with a mean ± SE value of 5.134 ± 1.822 ppb. This result is higher than results detected by Coffey et al. (2009) and lower than Breitholtz-Emanuelsson et al. (1993). In milk powder samples, OTA levels were detected in a range of 3.9 to 10 ppb with a mean ± SE value of 6.925±0.848 ppb (Table 5). This result is higher than results detected by Kabak (2012). OTA levels were detected in roomy cheese samples in a range of 3 - 4.8 ppb with a mean ± SE value of 3.811 ± 0.243 ppb, this result is nearly similar to Awad et al. (2012). OTA levels were detected in kariesh cheese samples in a range of 2 – 5.2 ppb with a mean ± SE value of 3.137± 0.441 ppb. This result is higher than Hussein (2013) Presence of OTA in cheese may refer to use of contaminated milk with toxin carried over from animals fed with contaminated feed (Monaci and Palmisano, 2004) or cheese contamination with OTA producing species as P.verrucosum and P.nordicum (Cabañes et al., 2010).

Table 5: Ochratoxin A (OTA(concentration in different examined samples

Samples types	Samples number	Positive Range (ppb)				Mean of level ± SE
		No.	%	Min	Max	
Raw milk	10	7	70	0.34	13	5.134±1.822
Milk powder	10	8	80	3.9	10	6.925±0.848
Roomy cheese	10	9	90	3	4.8	3.811±0.243
Kariesh cheese	10	8	80	2	5.2	3.137±0.441
Total	40	32	80	0.34	13	4.711±0.514

The highest incidence of OTA recorded in roomy cheese may refer to unhygienic conditions in package or storage which facilitate mould growth on cheese surface and subsequently toxin production in favorable conditions. Significant differences were detected between concentrations of OTA in different milk products (p=0.037). Presence of OTA in high concentration (0.34-13ppb) with mean 4.711±0.514 ppb (Table 5), may refer to presence of OTA with high level in animal feed especially that animal feed in Egypt depends on concentrated diet which change OTA degradation ability in rumen. About 35% of positive samples (that contain different levels of OTA) didn't contain OTA producing moulds, this may refer to disappear of producing mould during processing of dairy products or / and carry over from contaminated animal feed.

In conclusion, Presence of moulds and mycotoxins in milk and different dairy products with high percentage and high levels indicates public health hazards. So strict hygienic conditions in milk production, controlled hygienic conditions in dairy products factories, strict laws that prevent mycotoxin contaminated products reach to consumer, using advanced methods for mycotoxin prevention and control to protect human and animal life in addition to making more researches on mycotoxins and methods of

their prevention, should be considered.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR'S CONTRIBUTION

The 1st author (GY) designed the experiment and revised the manuscript. The 2nd author (DI) collected milk and dairy product samples, carried out the conventional culturing and mycotoxin detection, analyzed data, wrote the paper. The 3rd author (AA) partly wrote the manuscript and took the responsibility of correspondence to the journal. The 4th auhtor (MME) revised the manuscript. All authors approved the final version of the manuscript for publication.

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