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FUNGAL CONTAMINATION AND PREVALENCE OF AFLATOXIN M1 AND B1 IN MILK AND INFANT FORMULA

BY

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SUMMARY

This study comprises mycological and mycotoxicological investigations of commercial milk and infant formulas. A total of 300 random samples of milk (loose, UHT), milk powder and infant formula were collected from Cairo retail markets and subjected to fungal count and aflatoxins determination. Results showed that the fungal count was $2.7\pm0.42x10^5$ and $1.9\pm0.32x10^2$cfu/g in loose and powdered milk respectively, but was not detected in UHT milk and infant formulas. The highest mean aflatoxin M1 content was found in loose milk samples (35.9±3.3ng/kg), and the lowest was in infant formula samples (6.1±0.9ng/kg). The aflatoxin M1 contents in 36.7% of loose milk samples were higher than the maximum tolerance limit (50 ng/kg) specified in the Egyptian standards. Based on the aflatoxin M1 content in milk the calculated aflatoxin B1 in animal feeding stuffs were below the acceptable limits i.e. 5 μg/kg. It is recommended that the presence of fungal, and mycotoxins in milk should be mentioned throughout the chain of milk production until it reach the consumer in order to avoid consumer exposure to this hazardous contaminants. Also, regulations should cover the fungal contamination in milk; while those of mycological content in milk and feedstuffs should be complement.

Key words: aflatoxin; fungi; infant formula; milk; feedstuffs.

INTRODUCTION

Milk is an important nutrient for infants, children, convalescents and old people (Atasever *et al.*, 2010). However milk a highly perishable product (Tchekessi *et al.*, 2014), and it serves as an excellent growth medium for a wide range of microorganisms. The microbiological quality of milk and dairy products is influenced by the initial flora of raw milk, the processing conditions, and post-heat treatment contamination (Varga, 2007). Also, infant, follow-up and powdered formula as well as human milk fortifiers are to be distinguished from ready-to-feed liquid formula that have been commercially sterilized. As dehydrated products, it is not possible to produce sterilized powdered formula using current technologies but the products contain low levels of microorganisms. Thus, their microbiological safety requires strict adherence to good hygienic practices during both manufacture and use (CAC/RCP, 2008).

Although the microorganisms in infant milk powder cannot grow due to its low moisture content and have no direct role in their spoilage, their occurrence is of great significance and serves as an index of hygienic standards maintained during their production, processing and handling (Rajput *et al.*, 2009). Fungi influence the biochemical characteristics and quality of the product and may create hazard to human health (Parihar and Parihar, 2008). Rajput *et al.* (2009) found that all samples of infant milk powdered formulas contained <5±1.0 cfu/g of molds and yeasts, indicating their acceptable hygienic conditions and offers no risk for human health. The Egyptian Standards (ES:1648/ 2005) specify a maximum level of fungal count in milk powder to be 10 cfu/g, but it is not mentioned in raw milk standard (ES: 0154-01/2005) and the standard for the UHT milk (ES: 1623/2005).

Milk and milk products are considered a suitable medium for the growth of several fungi such as *Aspergillus flavus, A. parasiticus* and *A. nomius*, producing the potent *i.e.* aflatoxins. Thus, milk and dairy products are always at risk of being contaminated with aflatoxin M1 (AFM1) (Amer and Ibrahim, 2010). According to the Egyptian Standards (ES: 7136/2010), the maximum level of AFM1 in raw and heat-treated milk should not exceed 50 ng/kg, and not more than 25 ng/kg in infant and follow on milk formula. Several researchers have reported a positive correlation between the amount of aflatoxin B1 (AFB1) in the feed consumed by the animals and levels of aflatoxin M1 (AFM1) in milk (Hosny *et al.*, 2014). It's presence in milk is considered as a potential risk for human health because of its carcinogenicity potential and thus a need of regular monitoring in milk and dairy products (Mulunda *et al.*, 2013; Ali *et al.*, 2014). For various reasons many regions are obliged to feed dairy animals on stored forage or Industrially produced pellets. Therefore it is important to reduce the occurrence of toxins (AFB1) in feedstuff and to take prophylactic measures to counteract the factors enhancing toxin production (Jankovic *et al.*, 2009). However, no regulations were laid down for the dairy animals' feedstuff AFB1 specifying the level in the Egyptian Standards. Therefore, European Commission Directive (EC/100/2003) was followed, 5µg/kg which specify a maximum AFB1 level of animal feed. Following the withdrawal of contaminated source, AFM1 concentration in the milk decreased to an undetectable level within 72 h (Rahimi *et al.*, 2009). AFM1 which is subsequently secreted in milk of lactating cows is quite stable to normal milk processing methods and may persist in the final milk products for human consumption. The amount of AFM1 toxins excreted in milk usually represent 1-3% of the AFB1 in the animal feed consumed, but higher values (~ 6%) have been reported (Jouany and Diaz, 2005; Hosny *et al.*, 2014).

Limited studies have been cited on the micological properties of milk and infant formulas, and some of these studies were concerned only with pathogenic fungi. The present study investigated the fungal contamination of fluid and powdered milk in addition to infant formula and susceptibility content of AFM1 and AFB1 in these products.

MATERIALS AND METHODS

- **Sampling**

A total number of 300 full cream milk samples were analyzed for fungal (molds and yeasts) and AFM1 contamination (Table 1). All samples were randomly purchased from Egyptian retail markets in Cairo governorate within the year 2014. The samples were transported to the laboratory in an insulated container at about 4°C and analyzed upon arrival. Each sample was divided into two portions, one for fungal evaluation and the other for AFM1 detection.

- **Determination of fungi**

The milk samples were diluted to prepare 10^{-1} dilution in a sterile stomacher bags (Seward Stomacher 3500, Lab system, England) for 2 min, and then diluted successively up to 10^{-6} in 0.1% sterile buffered peptone solution (Oxoid CM9) (Soriano *et al.*, 2002). The serial dilutions were plated in duplicate using pour plate technique. Fungal cell count was done using Sabouraud dextrose agar medium (Oxoid CM41) containing 0.05 mg of chloramphenicol (Oxoid SR78) per ml and then incubated at 25°C for 5 days (El-Diasty and Salem, 2009).

- **Determination of aflatoxin M1**

Powdered milk samples were reconstituted in distilled water before analysis following the individual direction of each brand. The reconstituted milk and fluid milk samples were defatted by centrifuging at 2000g for 5 min. The defatted milk samples were subjected to the competitive direct ELISA test kit for AFM1 (Neogen, Veratox, UK) as described by Vagef and Mahmoudi (2013). The test was read and calculated in MRX micro well reader (Dynatech Laboratories, UK) with Software Version 1.2 to values in ng/kg.

Table (1): Number and type of samples analyzed.

Sample type	Number of samples	Sample type	Number of samples
Loose	**60**	**powdered**	**60**
• Location 1	20	• Brand 1	20
• Location 2	20	• Brand 2	20
• Location 3	20	• Brand 3	20
UHT	**120**	**Infant formula**	**60**
• Brand 1	20	• From birth on	20
• Brand 2	20	• From 6 to 12 month	20
• Brand 3	20	• From 1 to 3 years	20
• Brand 4	20		
• Brand 5	20		
• Brand 6	20		

- **Calculation of aflatoxin B1**

The aflatoxin B1 content in the animal feed was calculated from the M1 content of milk samples according to the following equation (Atasever *et al.*, 2010):

$$AFB1\ (\mu g/kg) = \frac{AFM1\ (ng/kg) \times 100}{1.6 \times 1000}$$

- **Statistical analysis**

The individual observations were analyzed and expressed in terms of mean ± standard error (SE). Analysis results were processed using statistical software (IBM-SPSS, 19; USA) that permitted to make analysis of variance (ANOVA) for comparison of means. A probability level of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Results recorded in Table (2) showed that the fungal counts in loose and powdered milk were $2.7 \pm 0.42 \times 10^5$ and $1.9 \pm 0.32 \times 10^2$ cfu/g, respectively, while was not detected in UHT milk and infant formula. The frequency distribution of fungal count in the positive samples ranged between 10^1-$<10^6$ cfu/g in loose milk and 10^1-$<10^3$ cfu/g in powdered milk. The mean count in only loose milk samples showed a statistically significant difference ($p<0.05$) from the other 4 groups, whereas, these later groups were not significantly different ($p>0.05$) in between.

It was observed that fungal counts ranged widely in all types of milk samples. These results are in agreement with those reported by El-Diasty and El-Kaseh (2009); Rajput *et al.* (2009).

Therefore, presence of fungi in milk, molds may create hazard to one's health, produce an allergen and an irritant to human health (Parihar and Parihar, 2008).

Obviously, it is important to prevent mold growth to avoid toxin production through preventing the natural contamination of raw materials.

In case of powdered milk and infant formula, storage at elevated relative humidity for 7 days showed a slight increase in the fungal counts but the toxin levels were unaffected (Aidoo *et al.*, 2011). On the other hand, Majeed *et al.* (2002) found that, the counts of molds and yeasts of dried milk samples were very little in all samples and can be insignificant.

Afroz *et al.* (2013) revealed the contamination of all the milk samples with yeasts and molds to the lack of hygiene in production and post-processing.

However, in developing countries, where climatic and crop storage conditions are frequently conducive to fungal growth and mycotoxin production, the population relies mostly on subsistence farming or on unregulated local markets (Awad *et al.*, 2014).

Table (2): Mycological quality of milk samples.

Milk samples	n	Positive samples		Range		Mean±SE
		No.	%	Min.	Max.	
Loose	**60**	**60**	**100**	**$4.1x10^2$**	**$9.4x10^5$**	**$2.7\pm0.42x10^5$ [a]**
• Location1	20	20	100	$4.1x10^2$	$9.3x10^5$	$2.0\pm0.71x10^5$ [a]
• Location2	20	20	100	$5.8x10^2$	$8.2x10^5$	$2.8\pm0.76x10^5$ [a]
• Location3	20	20	100	$3.7x10^3$	$9.4x10^5$	$3.4\pm0.72x10^5$ [a]
UHT	**120**	**0**	**0**	**<10**	**<10**	**<10 [b]**
• Brand1	20	0	0	<10	<10	<10 [b]
• Brand2	20	0	0	<10	<10	<10 [b]
• Brand3	20	0	0	<10	<10	<10 [b]
• Brand4	20	0	0	<10	<10	<10 [b]
• Brand5	20	0	0	<10	<10	<10 [b]
• Brand6	20	0	0	<10	<10	<10 [b]
Powdered	**60**	**24**	**40**	**$3.1x10^2$**	**$7.2x10^2$**	**$1.9\pm0.32x10^2$ [c]**
• Brand1	20	8	40	$3.7x10^2$	$7.2x10^2$	$1.9\pm0.58x10^2$ [c]
• Brand2	20	10	50	$3.1x10^2$	$5.0x10^2$	$2.0\pm0.48x10^2$ [c]
• Brand3	20	6	30	$5.6x10^2$	$6.9x10^2$	$1.8\pm0.65x10^2$ [c]
Infant formula	**60**	**0**	**0**	**<10**	**<10**	**<10 [d]**
• From birth on	20	0	0	<10	<10	<10 [d]
• From6 to12 month	20	0	0	<10	<10	<10 [d]
• From 1 to 3 years	20	0	0	<10	<10	<10 [d]

• Mean ± SE with the same symbol were not significantly different (p>0.05).

Storage and the favorable climate conditions can favor growth of mold from the genus of *Aspergillus* generating aflatoxins in feed and consequently its occurrence in milk (Polovinski *et al.*, 2008).

The occurrence and the distribution of AFM1 concentration in various milk samples are presented in Table (3). It was found above the detectable levels in 73.3, 48.3, 60, 46.6% of loose, UHT, powdered and infant formula milk samples, respectively. The highest mean concentration was 35.9 ± 3.3 ng/kg in loose milk samples and the lowest was 6.1 ± 0.9 ng/kg in infant formula samples. In 36.7 of loose milk samples, the AFM1 levels were higher than the maximum acceptable limits namely: 50 ng/kg, while all infant formula, UHT and powdered milk samples were within the acceptable limits specified in the Egyptian Standards (ES: 7136/ 2010), namely: 50 ng/kg for milk and 25 ng/kg for infant formula. The frequency distribution of positive samples ranged between 5 and 72 ng/kg. The research hypothesis is that the mean concentration of AFM1 in milk samples was significantly lower ($p<0.05$) than the reference range is accepted. Also, the mean concentrations between the 5 brands were significantly different ($p=0$).

These results are in parallel with the findings of some previous reports (Al-Zuheir and Abo Omar, 2012; Hosny *et al.*, 2014).

Factors such as season, time consumption and improper handling of food can be involved in the presence of AFM1 in milk.

There are some contradictions in literature relating to the effect of heat treatment on the aflatoxin contents of milk. Some studies indicate that sterilization treatments had no effect on the content of AFM1 in milk (Prandini *et al.*, 2009; Fallah, 2010). However, some reports showed that content of AFM1 was degraded in heated milk depending on time and temperature combination of heat treatment applied (Şanli *et al.*, 2012).

Table (3): Occurrence and frequency distribution of AFM1 in examined milk samples (ng/kg) concentration.

Milk samples	n	Positive samples		Range		Refe-rence range[1]	Mean ± SE	Exceeding ES		Frequency distribution			
		No.	%	Min	Max.			No.	%	<5	5-25	26-50	>50
Loose	**60**	**44**	**73.3**	**18**	**72**	**50**	**35.9±3.3**	**22**	**36.7**	**16**	**6**	**16**	**22**
• **Location1**	20	13	65	21	70	50	33.75±6.19	8	40	7	1	4	8
• **Location2**	20	16	80	19	72	50	39.05±5.51	7	35	4	2	7	7
• **Location3**	20	15	75	18	71	50	34.90±5.66	7	35	5	3	5	7
UHT	**120**	**58**	**48.3**	**5**	**50**	**50**	**10.22±1.25**	**0**	**0**	**62**	**38**	**20**	**0**
• **Brand1**	20	6	30	12	50	50	8.05±3.29	0	0	14	3	3	0
• **Brand2**	20	10	50	9	48	50	10.75±3.10	0	0	10	6	4	0
• **Brand3**	20	8	40	5	39	50	6.05±2.35	0	0	12	6	2	0
• **Brand4**	20	11	55	10	49	50	12.45±3.14	0	0	9	7	4	0
• **Brand5**	20	12	60	5	48	50	12.7±3.35	0	0	8	8	4	0
• **Brand6**	20	11	55	6	49	50	11.35±3.14	0	0	9	8	3	0
Powder	**60**	**36**	**60**	**5**	**44**	**50**	**12.9±1.8**	**0**	**0**	**24**	**23**	**13**	**0**
• **Brand1**	20	13	65	5	41	50	14.55±3.23	0	0	7	9	4	0
• **Brand2**	20	8	40	5	44	50	8.35±13.45	0	0	12	4	4	0
• **Brand3**	20	15	75	6	43	50	15.7±2.89	0	0	5	10	5	0
Infant formula	**60**	**28**	**46.6**	**5**	**22**	**25**	**6.1±0.9**	**0**	**0**	**32**	**28**	**0**	**0**
• **From birth on**	20	4	20	11	21	25	3.65±1.72	0	0	16	4	0	0
• **From6 to12 month**	20	14	70	5	22	25	7.80±1.55	0	0	6	14	0	0
• **From 1 to 3 years**	20	10	50	6	21	25	6.95±1.74	0	0	10	10	0	0

1: According to Egyptian standards (ES:7136/2010). *: p-value between groups = 0.

Many researchers have reported a positive correlation between the amount of AFB1 in feed consumed by animals and levels of AFM1 in milk (Amer and Ibrahim, 2010; Abdallah *et al.*, 2012).

A calculated AFB1 content in cattle feedstuffs based on AFM1 concentration in milk samples was illustrated in Table (4). Similar to the mentioned AFM1 results, the numbers and percentages of AFB1 positive samples were similar to that found for AFM1. It can be seen from the results that the mean content of feed with AFB1 in cattle feed ranged between 0.38 ± 0.06 and 2.24 ± 0.20 µg/kg. None of these results exceeded legal limit (5 µg/kg) in complete feedstuffs for dairy animals by the European Commission Directive (EC/100/2003) where the Egyptian standards did not include this case.

Making silage in good conditions anaerobiosis and low pH usually prevents the development of fungi. The presence of oxygen at the cut edge of the silage or in the silo may favor the growth of fungi (Yiannikouris and Jouany, 2002). These fungal species can synthesize and excrete into environment secondary metabolites of various chemical compositions that worsen silage quality, which becomes hazardous for cattle health (Baliukonienė *et al.*, 2012).

Moreover, fungi present in haystacks may easily produce toxins in appropriate storage conditions. Following the consumption of highly contaminated feed with AFB1, conversion of AFB1 to AFM1 takes place in the liver and leads to elevated levels of AFM1 in the milk. Therefore, it is important to reduce the occurrence of toxins (AFB1) in feedstuff and take prophylactic measures to prevent factors enhancing toxin production (Amer and Ibrahim, 2010; Al-Zuheir and Abo Omar, 2012).

Table (4): Calculated aflatoxin B1 (µg/kg) content in cattle feedstuffs based on AFM1 content in milk samples.

Milk samples	n	Positive samples		Range		Reference range[1]	Mean±SE
		No.	%	Min.	Max.		
Loose	**60**	**44**	**73.3**	**1.12**	**4.50**	**5**	**2.24±0.20**
• Location 1	20	13	65	1.31	4.37	5	2.10±0.38
• Location 2	20	16	80	1.18	4.50	5	2.44±0.34
• Location 3	20	15	75	1.12	4.43	5	2.18±0.35
UHT	**120**	**58**	**48.3**	**0.31**	**3.12**	**5**	**0.63±0.07**
• Brand 1	20	6	30	0.75	3.12	5	0.50±0.20
• Brand 2	20	10	50	0.56	3	5	0.67±0.19
• Brand 3	20	8	40	0.31	2.43	5	0.37±0.14
• Brand 4	20	11	55	0.62	3.06	5	0.77±0.19
• Brand 5	20	12	60	0.31	3	5	0.79±0.20
• Brand 6	20	11	55	0.37	3.06	5	0.70±0.19
Powdered	**60**	**36**	**60**	**0.31**	**2.75**	**5**	**0.80±0.11**
• Brand 1	20	13	65	0.31	2.56	5	0.90±0.20
• Brand 2	20	8	40	0.31	2.75	5	0.52±0.18
• Brand 3	20	15	75	0.37	2.68	5	0.98±0.18
Infant formula	**60**	**28**	**46.6**	**0.31**	**1.37**	**5**	**0.38±0.06**
• From birth on	20	4	20	0.68	1.31	5	0.22±0.10
• From 6 to12 month	20	14	70	0.31	1.37	5	0.48±0.09
• From 1 to 3 years	20	10	50	0.37	1.31	5	0.43±0.10

1: According to the European Commission Directive (EC/100/2003) on undesirable substances in animal feed.

The most important factors affecting the amount of AFB1 occurrence in feed was undoubtedly temperature and moisture. The toxin producing fungi such as *Aspergillus flavous* and *A. parasiticus* species show enormous growth in feeds having water contents between 13 and 18% and environmental moisture between 50 and 60%. Furthermore, these molds can produce the toxin under conditions of 25°C and 85-90% relative humidity (Bakirci, 2001; Muhammad *et al.*, 2010).

The amount of AFB1 in animal feed can be minimized by taking care of cultural phases, including harvest and storage practices that present critical points for fungal growth and mycotoxin production (Prandini *et al.*, 2009).

CONCLUSION

To ensure an efficient protection of public health, it is essential to choose a good quality of milk as well as regular monitoring of the mycological and mycotoxic quality of milk.

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التلوث بالفطريات و مدى تواجد بقايا الأفلاتوكسينات فى اللبن وتركيبات ألبان الأطفال

تشتمل هذه الدراسة على مناقشة تواجد الفطريات وسمومها فى عينات الألبان وتركيبات ألبان الأطفال المتواجدة فى الأسواق. تم جمع ٣٠٠ عينه لبن (غير معبأ ومعقم)، لبن جاف وتركيبات ألبان الأطفال عشوائيا من الأسواق فى القاهرة وتقدير العد الكلى للفطريات والأفلاتوكسين. ولقد أوضحت النتائج أن العد الكلى للفطريات كان ٢.٧ ± ٤.٢ X ١٠^{٥} و ١.٩ ± ٣.٢ X ١٠^{٢} خلية/جرام فى الألبان الغير معبأة والجافة، على الترتيب، بينما كانت الألبان المعقمة وتركيبات ألبان الأطفال خالية من أى أعداد. كذلك سجلت عينات اللبن الغير معبأ تواجد أعلى تركيز من أفلاتوكسين م١ (٣٥.٩ ± ٣.٣ نانوجرام/كجم) وعينات تركيبات ألبان الأطفال إحتوت على أقل تركيز (٦.١ ± ٠.٩ نانوجرام/كجم).

كما أوضحت النتائج أن ٣٦.٧٪ من عينات اللبن الغير معبأ تحتوى على نسبه من أفلاتوكسين م١ أعلى من الحدود المسموح بها (٥٠ نانوجرام /كجم) طبقا للمواصفات القياسيه المصريه. وبالرجوع إلى محتوى أفلاتوكسين م١ فى اللبن، كانت أفلاتوكسين ب١ المحسوبه فى أعلاف الحيوانات المنتجة للبن فى الحدود المسموح بها (٥ ميكروجرام/كجم).

وخلصت الدراسة إلى أن عمليات التصنيع تعكس مستوى التلوث بالفطريات والأفلاتوكسينات والتى تحتاج لمتابعة مستمرة لضمان سلامة المستهلك ولتقليل معدلات التعرض لتلك الملوثات. كذلك فإن المواصفات القياسية يجب أن تشمل حدود الفطريات الملوثة للبن، وكذلك تلك المتعلقة بالسموم الفطرية فى الألبان وأعلاف الحيونات المنتجة للبن يجب أن تتطابق.