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Aflatoxin in Infant Milk Formulae: Potential Mutagenicity and Management

G.M. Abdel-Fattah[1]; M. I. Soliman[1]; E. E. Hafez[2] and H. S. El-Sherbeiny[1]

[1]*Botany Department, Faculty of Science, Mansoura University*

[2]*Molecular Plant Pathology Department, City for Science and Technology Applications*

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Abstract: (The main purpose of this study was to investigate the aflatoxin M1 (AFM1) presence in infant milk formulae and aimed also to study its cytotoxic and genotoxic effect using a test plant, *Allium cepa* and also to manage AFM1 production using different essential oils of some Egyptian higher plants. The study was conducted in Faculty of Science, Mansoura University. A total of 10 samples were collected from different pharmacies to screen the presence of AFM1 in three exposure times, 0, 15 and 30 days from the opening of the formulae using the enzyme-linked immunosorbent assay (**ELISA**). The amount of AFM1 ranged from 887 to 1125 *p*g/ml. The study also aimed to isolate the fungi found in the baby milk responsible for the AFM1 production. *Aspergillus flavus*, *A. parsiticus* and *Pencillium* species. are some of the mycoflora isolated from the studied formulae. After that, isolation of the AFM1 using GC-MS (Gas Chromatography Mass Spectrometry) was conducted in central laboratory, the City for Scientific Research and Technology Applications.

keywords: Aflatoxin M1,ELISA,Essential oils,Aspergillus flavus

1.Introduction

Infant formula is counted to be an efficient alternative to breast milk and is manipulated and formulated to imitate the nutritional component of breast milk [1]. In the 1960s, the concern for mycotoxin contamination in dairy products began, with the 1st reported cases of contamination by aflatoxin M1 (AFM1), the aflatoxin B1 (AFB1) metabolite which secreted in the milk after production in the animal rumen. Mycotoxins are toxic metabolic by-products of molds present on all agricultural merchandises all over the world. Secondary metabolites are not essential in the normal metabolic functions of the fungus (e.g. phytotoxins and antibiotics) unlike the primary metabolites (sugars, amino acids and other substances), [2]. There are around 400 reported mycotoxins currently. Under natural conditions, these compounds occur in feed as well as in food. Aflatoxins, trichothecenes, fumonisins, zearalenone, ochratoxin and ergot alkaloids are some of the most famous mycotoxins known.

Mycotoxins are produced by some species of fungi from the genera Aspergillus, Penicillium, Fusarium, and Claviceps. Also each strain can produce more than one mycotoxin [3]. After animals ingest polluted food, mycotoxins are metabolized and transferred to animal products, such as milk or meat, which is considered a big risk to human health [4]. The most studied mycotoxin in milk is aflatoxin M1. Aflatoxins are toxic carcinogenic compounds that interpose with the immune system and commonly produced by certain fungal species (Aspergillus flavus and Aspergillus parasiticus) [5] that can grow in decaying vegetation, hay soil, as well as grains specifically in hot and humid weather [6]. AFs have many pernicious health effects as they are carcinogenic, hepatotoxic, teratogenic, mutagenic, and they have also immune-suppressive effects [7]. AFs caused acute toxicity and chronic carcinogenicity in human and animal populations [7]. International Agency of Research on Cancer (IARC) classified AFB1 as a Group 1 carcinogen that prosecute hepatocellular carcinoma (HCC) in individuals exposed to this kind of aflatoxin, whereas AFM1 is listed in Group 2B (most probably carcinogenic to humans) [8.[

AFM1 in Egypt

A study was conducted by Tomerak, et al. [9] on 150 mothers and infants that were

exclusively breastfed. The weights' standard deviation of infants was documented at birth and at 6 months. 63 % of mothers had AFM1-positive breast milk samples. Hosny et al. [10] examined some of dairy products for the presence of AFM1 in Alexandria local market, Egypt. The maximum levels of the contamination were in raw and pasteurized milk whilst minimum levels were detected in infant milk.

Another study was conducted by Shaker and Elsharkawy [11] to evaluate the amount of AFM1 in raw, pasteurized and ultra-heat treated (UHT) buffalo milk consumed in upper Egypt using ELISA technique. All the tested samples were positive for AFM1, that were above the Egyptian regulation's limits (5.00 ng/L.(

Factors Influence Aflatoxin M1 Milk Contamination

An assortment of factors favor the production of mycotoxins has been discovered. Those can be classified as physical, chemical, and biological factors. Environmental conditions like relative humidity, temperature and insect infestation they are considered as physical factors while the use of fungicides or fertilizers, thy are chemical factors. Biological factors depend on the interactions between the substrate and the colonizing toxigenic fungi. Actually, some plant species are more susceptible to colonization where environmental conditions may increase the sensitivity of others [12 .[Materials and methods 1Samples preparation

The commercial names of the collected samples were as follow, Aptamil 1, Bebelac 1, Hero Baby 1, Celia 1, Nan 1, Swisslac Premium, S-26 Gold 1, Primavita 1, Liptomlik 1, and Similac 1. The infant formulae were aseptically transported to the laboratory for the screening of aflatoxin M1 and further studies .

.2Detection of Aflatoxin M1(AFM1(

The quantitative analysis of AFM1 in examined samples (infant formula) was performed by competitive ELISA (AFM1 2000 ELISA kit, No. 961AFLMUS-96). The study was conducted after opening the formula boxes (0 time), after fifteen days from the opening and after thirty days for all samples. Each sample was prepared by adding six formula scoops to 200 ml water. Samples placed at 4o C for 1 – 2 hours then to enhance the separation of the upper fatty layer, samples were centrifuged at 2,000 rpm for 5 minutes. After the upper fatty layer was removed by centrifugation, lower plasma had used in the assay procedure as follow.

.1Before use, reagents were brought to the room temperature. Washing out the contents with a distilled water to picket the PBS-Tween Q.S then complete to 1 liter with distilled water and store in the refrigerator till use.

.2To avoid the entry of moisture, the unused wells returned to their bag and re-sealed.

200 .3µl (micro liter) of the sample was taken and diluted into each mixing well.

100 .4µl of standards and samples were dispensed (using a fresh pipette tip) into the appropriate wells and mixed properly .

100 .5µl of the mixture was transferred to the corresponding Antibody Coated Well using a multichannel pipette, and then incubated at room temperature for 10 minutes.

.6A sufficient conjugate (120 µl per standard/sample) was placed in a dock and 100 µl of the conjugate was added to the wells that contain standard/sample. After that, samples were incubated at room temperature for 30 minutes.

.7The contents were decanted from the micro wells into a discard basin. The micro wells were washed by filling each with PBS-Tween wash buffer, then the wash was poured into a discard basin. This step of wash was repeated three times.

.8The volume needed from the Substrate Solution (1ml / strip or 120 ml/well) was measured and placed in a separate container. 100 µL was added to each micro well, then Incubated for 10 minutes. Wells were covered to avoid direct light.

.9The volume of Stop Solution required (1ml / strip or 120 ml/well) was measured and placed in a separate container. 100 µl was added in the same sequence and at the same time with the Substrate Solution.

.10With a microtiter plate reader using a 450 nm filter, the optical density (OD) of each microwell was read and recorded. The results were tabulated.

.3Isolation of molds from the infant formula

Using the serial dilution method, the collected samples were processed for analysis and followed by the pour-plate technique [13]. Ten millimeters of each milk sample in the three studied times (0, 15 and 30 days) were transferred aseptically into a sterile blender jar, to which 90 ml of 1% peptone water were added and homogenized in a sterile warring blender for 2 minutes. The dilutions of 10-1 and 10-2 were prepared and one milliliter quantities from each dilution tube were inoculated separately in Petri dishes with a sterilized potato dextrose agar (PDA) medium in three replicates. The prepared plates were incubated upside down in an incubator at 28°C±2°C. After 7days, the number of Colony Forming units developed on PDA plates were counted. To obtain the total fungal count (TFC), the number of colonies was multiplied by the dilution factor and was recorded in Colony Forming Unit per milliliter (CFU / ml) [14.[

4Identification of the isolated molds

The isolated fungi were sub-cultured onto PDA using the streaking method and incubated at 27o C for 4 days. The isolates identified according to morphological structure individually by macro- and microscopic characteristics of the mold colonies at Botany department laboratory, Mansoura university. Macroscopically identification was assigned by predestining the colony countenance like; color, shape, size and hyphae while microscopically by a compound microscope with a digital camera to identify the conidial head, stigma and conidial ontogeny [15]. The fungal genera and species were identified according to the following references; (Raper and Thom [16], for Penicillium and Penicillium related species) and (Raper and Fennell [17], for Aspergillus species). Also, all isolated fungi were identified molecularly at the City for Scientific Research and Technology Applications.

The Effect of the Essential Oils on Fungal Growth and Aflatoxin Production

Antiaflatoxigenic efficacy and antifungal activity of five kinds of essential oils with different doses were determined using sterilized milk medium. The study was carried out on six of isolated fungal species from the infant formulae; A. niger, A. flavus, A. ochraceus, A.oryzae, A. sydowii and pencillium granulatum. The surfactant (25% Tween in sterile water) was added to oils. Different doses of each oil (1, 2, and 4%) were obtained from Faculty of Agricultural, Cairo University and added to the media. Discs of 6 mm diameter of the toxigenic species were inoculated to the flasks with and incubated for 7 days at 25 ± 1°C. control was carried out with Tween 80. Three replicates of each concentration were performed. After incubation, the content of each flask was filtered using sterilized filter papers (Whatman, number 1) and the biomass of filtered mycelium was dried at 70°C for 4 days till their weights remain constant. The fresh and dry weight from each sample were measured. The filtrates of samples treated with 4%. oils and control were stored in -200 C for the ELISA test .

ELISA test was performed to detect the amount of AFM1 produced by some of the isolated fungal species from the infant formulae and also to measure their efficiency of AFM1 production after treatment with the essential oils (4%). The experiment was carried out on the filtrates of all samples as described in the detection of AFM1 in infant formulae.

Result

1ELISA screening for AFM1 in the infant milk formulae

The infant formulae were screened for the presence of AFM1 in different times; after the opening of the formulae (0 time) , after 15 days and after 30 days. As shown in Table (1), all examined samples in all times have AFM1 with different concentrations. In the first time, AFM1 amount ranged from 887 pg/ml in Bebelac 1 (the lowest value) to 989 pg/ml in Liptomilk 1 (the highest value). After 15 days from opening the formulae, the highest AFM1 concentration was 1060 pg/ml in Liptomilk and lowest amount was 968 pg/ml in Nan 1. As represented in the table, AFM1. after 30 days ranged from 981 pg/ml in HeroBaby 1 to 1070 pg/ml in Nan 1.

.2Identification and Enumeration of toatl fungal counts

According to morphological and molecular identification of the molds isolated from the infant milk formulae in all screened times, the isolated fungi identified as follow: Aspergillius

niger, A. flavus, A. ochraceus, A. oryzae, A. sydowii, Pencillium granulatum and P. chryogenum.

Table (1): ELISA screening for AFM1 (*p*g/ml) in examined infant formulae

Infant Formula	Exposure Time (days)		
	Zero	15	30
Aptamil 1	954	1035	1035
Bebelac 1	887	968	1032
Hero Baby 1	962	980	981
Celia 1	960	970	975
Nan 1	937	968	1070
Swisslac Premium	931	1015	1043
S-26 Gold 1	950	968	1036
Primavita 1	955	1065	1124
Liptomlik 1	989	1060	1125
Similac 1	982	987	1012

All examined samples showed fungal growth with different species and different counts. As represented in **Table (2)**, the total fungal count (TFC) at zero time ranged from 80 CFU/g (the lowest value) in Bebelac 1 to 150 CFU/g (the highest value) in Swisslac Premium 1 where CFU stands for Colony Forming Units. After 15 days from opening the formulae, as shown in **Table (3)**; TFC were ranged from 740 CFU/g in Hero Baby 1 to 1180 CFU/g in S.26 Gold 1. After 30 days, according to **Table (4)**; the lowest total fungal counts were shown in Similac that was 1720 CFU/g and the highest TFC were shown in Celia 1 which was 2220 CFU/g.

3. ELISA screening for AFM1 concentration in some of isolated molds treated with essential oils (4%)

ELISA test was performed to measure the amount of AFM1 produced in some of the fungi isolated from the infant mlik formulae in different times (*Aspergillus niger, A. flavus, A. oryzae, A. occhraceus, pencillium granulatum and A. sydowii*) and also to measure the effect of some of the essential oils (4%) on AFM1 production from these fungi. The essential oils used in this study were from higher Egyptain plants, mint (*Mentha viridis),* sage *(Salvia officianalis),* wheat germ oil *(Triticum vulgare),* anise *(Pimipinella anisum) and* caraway *(Carum carvi)* and all of them inhibited the growth of the isolated fungi and reduce the amount of AFM1 production with different proportions.

According to the result represented in **Table (5)**, the concentration of AFM1 in *A. niger* was 1625 *p*g/ml and this value reduced when essential oils were added to the growth media where the highest effect was recorded from *S. officinalis* and *Carum carvi* (1162.5 *p*g/ml) and the lowest effect was from *M. viridis*. For *A. flavus*, the most essential oil that showed partial inhibition for AFM1 production was *P. anisum* (977 *p*g/ml) while the lowest effect was shown from *S. officinalis* (1030 *p*g/ml) although it had a great effect on AFM1 production from *A. oryzae* where the value reduced from 1623 to 975 *p*g/ ml while the lowest effect was shown from *T. vulgare*. For AFM1 of *A. occhreceus* and *Pencillium granulatum* , *P. anisum* showed the lowest effect (1412 , 1124 *p*g/ ml) where it was highly inhibited by *S. officinalis* (1021 *p*g/ml) in both molds. The AFM1 production from *A. sydowii* was highly reduced from 1362 to 987.5 pg/ml by *C. carvi* while *T. vulgare* showed the lowest effect (1237 *p*g/ml).

Table (2): Total fungal count and number of isolated fungi at zero time in infant milk formulae

Sample	TFC (CFU/ g) (x10)	No of isolated fungi
Aptamil 1	100	3
Bebelac 1	80	2
Hero Baby 1	90	2
Celia 1	110	3
Nan 1	100	2
Swisslac Premium	150	3
S-26 Gold 1	100	3
Primavita 1	90	2
Liptomlik 1	110	3
Similac 1	50	1

Table(3):Totalfungalcountandnumberofisolated fungi after 15 days in infant milk formulae

Sample	TFC (CFU/g) (x10)	No of isolated fungi
Aptamil 1	970	3
Bebelac 1	760	3
Hero Baby 1	740	3
Celia 1	840	4
Nan 1	1010	3
Swisslac Premium	1060	3
S-26 Gold 1	1180	4
Primavita 1	930	3
Liptomlik 1	980	3
Similac 1	970	2

Table (4): Total fungal count and number of isolated fungi after 30 days in infant milk formulae

Sample	TFC (CFU/g) (x10)	No of isolated fungi
Aptamil 1	1780	3
Bebelac 1	1870	3
Hero Baby 1	1750	3
Celia 1	2220	4
Nan 1	2050	3
Swisslac Premium	1860	3
S-26 Gold 1	1960	4
Primavita 1	1960	3
Liptomlik 1	2030	3
Similac 1	1720	3

Discussion

There is no doubt that humidity, food storage conditions, genetics of fungi and plants are some factors that obviously affect the production of mycotoxin. It is evident from our study and statistical analysis that all examined infant milk formulae in all screened times have aflatoxin M1 with different concentrations and also showed an increase in microbial population by increased storage time. In relation to many previously reported surveys worldwide for infant milk formula studied by **Baydar *et al.* [18]**, AFM1 was found in all examined infant formulae. This data also agrees with **Sharaf [19]**. These results are more prospective to be occurred in the parts of the world where improper storage and food handling are used and where few regulations exist to protect exposed populations. on the other side, AFM1 was detected by **Meucci *et al* [20]** and **Kabak [21]** but with a lower percentage while **El-Tras *et al.* [22]** and **Hoseny *et al* [23]** detected it by a higher percentage.

It is clear from all studies and researches that the AFM1 stability during handling or treatment and storage makes it critical. Studies confirmed that there were no considerable changes in the concentration of AFM1 after heat processing (pasteurization or boiling) or Ultra-high temperature **[24]**. Due to serious health concerns, maximum admissible standards have been put by many countries for AFM1 levels in milk and different dairy products and for AFB1 in animal feeds. However, the regulatory limits of permissible standards are influenced by economic considerations worldwide **[25] [26]**. In the present study AFM1 in all examined samples ranged from 887 – 1125 *p*g/ml or 887 to 1125 ng/kg these values exceed the regulatory limits by Egypt (0 ng /Kg) **[27] [28]**. AFM1 regulation limit in milk and milk products has been assigned by the US as 500 ng/kg **[29]**. Where the maximum acceptable limit by European Communities is 50 ng/kg of AFM1 **[30]** to protect consumers from contaminated milk. There is no evidence that any other manipulations of the contaminated milk change the level of AFM1 like concentrating or drying of the milk, cold storage or freezing, even the heat treatment (AFM1melting-point is 299^{o}C) and fermentation, **[31]**. Some urban countries reduce the maximum level of AFM1 to 10 ng/kg for infant foods as Austria and Switzerland **[30]**.

It is clear from the present study that the essential oils used with different doses have an inhibitory effect on the growth of the fungi isolated from the infant milk formulae also on the production of aflatoxin M1. These results agree with many searches; **Philippe *et al*. [32]** who studied the impact of six essential oils from *Cymbopogon citratus, Pimenta racemose, Ocimum gratissimum, Cinnamomum zeylanicum, Syzygium aromaticum* and *Zingiber officinale* on some dairy products and they showed a good inhibition on the growth of fungi and AFM1. **Yamina *et al.* [33]** also reported that the growth of *A. flavus* and its AFB1 production were inhibited by *Citrus maxima* essential oil. Our findings in this study also confirmed by **Abeer *et al*. [34]** that studied the essential oils effectiveness on *A. flavus* and *A. parasiticus* growth as well as on the AFB1 production. The results refer that the studied fungi are sensitive to the examined used essential oils and specifically to thyme and cinnamon.

Conclusion

The findings concluded from this study indicate that the examined infant milk samples marketed in Egypt contain aflatoxin M1 and detectable levels of molds. The contamination of milk and its derivatives by such carcinogenic toxin, as AFM1 is concerning especially during the early vulnerable stages of child development. There is no doubt that the dairy products are significant sources of calcium and are generally popular recommended, therefore,

they have to be investigated and examined continuously for aflatoxin and entero-pathogenic contamination since farming of animal food, controlling procedures in dairy factories and storage of samples. Finally, adding some of the essential oils extracted from higher plants to the infant milk formulae is recommended to control the AFM1 production.

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