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Mycobiota and Aflatoxins B1 and M1 Levels in Commercial and Home-made Dairy Desserts in Aswan City, Egypt

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ABSTRACT

Dairy desserts containing cereals are considered a proper medium for xerophilic fungi growth and aflatoxins production. In this study, a hundred fifty samples of different commercial dairy desserts (n=90) and homemade ones (n=60) were examined for total mold count and aflatoxins B1 and M1 levels in first day of production and after 3 days of cold storage at 5°C. The results, revealed high total mold count in all examined commercial samples especially Muhallabiah, 11.9x10^2 and 4.7x10^3 CFU/g in 1st and 3rd day of storage, respectively. The *Penicillium* spp. and *Aspergillus* spp. were the predominant isolates followed by *Alternaria* and However, aflatoxin B1 was detected in 70% of commercial Bellilah samples above the acceptable limits (2 ppb) and Aflatoxin M1 had exceeded the limits in 10% of each type of samples. In contrast, the homemade samples showed good quality and acceptable total mold count and Aflatoxin B1 and no Aflatoxin M1 (according to Egyptian Standard) up to 7 days of storage. In conclusion, using of good quality cereals and good quality milk in the manufacturing of dairy desserts is the keyword to obtain safe healthy dairy desserts for consumers.

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Introduction

Dairy desserts are the nutritious, covering various tastes and relatively inexpensive dairy food; they are usually prepared from ingredients, that the milk is the base constituent. These desserts Muhallabiah, Rice Milk and Bellila are almost consumed in Egypt by a wide range of people of all ages. So many restaurants submitted only for dairy desserts and designing special desserts for Egyptian taste. These desserts are manufactured from milk mixed with different cereals such as wheat, rice, and corn.

Fungi constitute large divergent group of microorganisms including numerous thousand species. Because of their heterotrophic nature and ability to adapt at wide range of environmental conditions, these fungi are encountered as actively growing on various commodities including foods. They are ubiquitous in nature. They may result in several types of spoilage. Mold as aerobic microorganism can grow on the surface of dairy desserts causing off-odors and flavors, as well as visible change in color and texture and raise pH of the product causing its spoilage (ledenbach and marshall, 2010). So, the total mold count is indicating shelf-life of the dairy desserts by great percentage and hygienic condition of food. Starting from cereals used in dairy desserts manufacturing to the sanitary conditions of handling and processing and packaging (Pitt and Hocking, 2009).

They are also responsible for many serious diseases through production of mycotoxins (Ostry *et al.*, 2017), which are secondary by-products of some mold genera produced under certain climatic conditions to achieve competitive advantage power to mold against other organisms. It is highly toxic to all microorganisms, animals and even to human being.

Among mycotoxins, aflatoxins are more potent, aflatoxin B1 (AfB1) is classified as group 1 human carcinogenic toxin and aflatoxin M1 as group 2B (IARC, 2002).

Aflatoxin B1 is one of about 18 types of aflatoxins (Bhat *et al.*, 2010) belonging mainly to *Aspergillus flavus* and *Aspergillus parasiticus* and may be *A. nomis* and *A. niger* (Elsanhoty *et al.*, 2014) and its main source to the human is cereal-based food. AfB1 is already known to be human hepatocellular carcinogenic agent according to IARC. European Commission Regulation (2006) determined the maximum limits of AfB1 in food by 2 ppb and 4 ppb to total aflatoxins. While FAO (1992) limitations are 10 ppb for total aflatoxins and 5 ppb in dairy feed for AfB1.

Aflatoxin M (AFM) is the epoxide intermediate form of

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AfB1 after liver metabolism. AFM is less harmful than AfB1 (but it is still toxic) and group 2B carcinogens (IARC, 2002). It can be found in milk and dairy desserts from one way, which is feeding of dairy animal on ration contaminated by mold spores and AfB1, which pass to the liver and converted to AFM then secreted with milk (Ogunade *et al*., 2018; Xiong *et al*., 2018). AFM as all aflatoxins is heat resistant and not degraded by the process of manufacturing. The AFM international permissible limit is 0.05 ppb according to FAO (1992).

Cereals are very susceptible to mold growth and mycotoxins production if stored carelessly at high relative humidity (Yin *et al*., 2008; Alim *et al*., 2018). These mycotoxins are not affected by the heat of pasteurization or processing (Bullerman and Bianchini, 2007). So can transfer from low-quality cereals used in dairy desserts manufacturing causing serious problems to human especially children. If the dairy animal was fed on high-level mycotoxins contaminated ration, the aflatoxin B1 will be converted in the animal liver and secreted with milk as aflatoxin M1 (Lopez *et al*., 2003; Fallah 2010; Xiong *et al*., 2018).

So if dairy desserts were manufactured from mixtures of contaminated milk with aflatoxin M1 come from animal feed on contaminated ration (Ogunade *et al*., 2018), and mixed with poor quality cereals (wheat, rice or corn) that were contaminated originally with molds. The produced dairy dessert may contain both aflatoxin M from milk and aflatoxin B from cereals, which means great health hazard on human.

Therefore, this work was designed to scan dairy desserts manufactured and sold in Aswan city markets for the presence of aflatoxin B1 and M1, and compare with homemade dairy desserts prepared by the authors.

Materials and methods

Commercial samples

A total of ninety random samples of dairy desserts (Bellilah, Rice Milk, and Muhallabiah) 30 each were collected from Aswan markets, Egypt in the period between June and September 2017.

Homemade samples (HM)

Raw milk was collected from bulk tank in dairy farm in Aswan. Acceptance tests were applied on raw milk, which include alcohol precipitation test (APT), total bacterial count (TBC), total mold count (TMC) and somatic cells count.

Cereals (wheat, rice, starch) used in homemade samples were purchased from supermarkets in Aswan. Both milk and 3 types of cereals individually were tested and proved negative for mold and aflatoxins. (Results not included)

Sixty (20 each) samples of Bellilah, Rice Milk and Muhallabiah were prepared in the laboratory of Food Safety Department at Aswan University by using boiled good quality raw milk and good quality grains and starch under acceptable sanitary conditions that mimic those in the ordinary kitchen as follow:

Bellilah (milk pudding including wheat)

Bellilah was prepared by addition of milk gradually to previously boiled wheat grains till the formation of suitable texture.

Rice Milk (milk pudding with rice)

Rice Milk was prepared by warming of rice up to 80°C then milk was added gradually till obtaining the end product.

Muhallabiah (milk pudding with starch or corn flour)

Muhallabiah obtained by dissolving corn flour (starch) in little amount of cold milk then added to warm milk with agitation till boiling and the poured in suitable cups.

All commercial samples as well as homemade ones were stored under the same condition at refrigeration 5°C and subjected to mycological examination individually for aflatoxin B1 and M1 after pooling each 3 samples in one for commercial samples and each 2 in one for homemade ones.

Mycological examination

Total mold count (TMC)

TMC was performed on day 0 of production and day 3 (end of shelf life) according to Pitt and Hocking (2009) using Dichloran 18% Glycerol Agar (DG18) medium. An amount of 1ml prepared sample was spread on surface of DG18 and incubated at 25°C for 3 to 5 days and then colonies were counted.

Isolation and identification of mold genera

Each separate colony was picked and inoculated into Czapek Dox medium at 25°C for 3 days and identified according to Pitt and Hocking (2009).

Detection of aflatoxins B1 and M1

Aflatoxins were detected by using HPLC (Agilent Technologies, Agilent 1260 Infinity HPLC with a Fluorescence Detector G1321B, USA) in the central laboratory of Agricultural Pesticide, Giza, Egypt. according to Herzallah (2009); Shuib *et al*. (2017).

Experimental conditions

Standard AFL: AFLS standard (B1, M1) was purchased from Dr. Ehrenstorfer, Augsburg, Germany.

HPLC Conditions

Column: Zorbax C18 XDB column with 5 μm particle size
Mobile phase: Acetonitrile: methanol: water (13:22:65 v/v/v)
Flow rate: 1.0 mL/min
Temperature: Ambient (25 ºC)
Detector: UV 360 and 440 nm
Injection volume: 20μL

Preparation of samples according to Herzallah (2009)

Extraction

A weight of 20 g sample was extracted with double volume of acetonitrile/ water mixer (9:1) by a mechanical shaker for 30 min.

Cleanup

Cleanup was done by adding 2 ml of obtained filtrate to an IMC (ISOLUTE TM Multimode Column, 3 ml capacity, 50 mg mass)

Derivatization

An aliquot (300 μl) of sample solution was transferred at once into a HPLC vial. Then mixed with n-Hexane and TFA (trifluoroacetic acid and kept for 5 min, to obtain separate layers. The desired quantity of aqueous layer was auto-injected into the HPLC column.

Determination of aflatoxins by HPLC

Aflatoxins were determined using an Agilent 1260 HPLC with a fluorescence detector, operated at an excitation and emission wavelength of 360 and 440 nm, respectively, and a 250 x 4.6 mm id Zorbax C18 XDB column with 5 µm particle size.

Results

Results achieved in Table 1 declared that TMC in Muhallabiah was the highest range $11.9x10^2$ and 70% of samples were contaminated by mold in first day of production, while 100 % of samples were contaminated at the end of shelf-life (day 3) with TMC of $4.7x10^3$.

Also in Table 1, Rice Milk samples were positive for mold (16.6 and 43.3%) in first and third day of production, respectively.

Table 2 showed the genera of mold isolated from dairy desserts in which *Penicillium* spp. were represented (37.8%), (38.5%) and 3.5%) in Bellilah, Rice Milk and Muhallabiah, respectively. While, *Aspergillus* spp. were in percentages of 24.3%, 22.7% and 48% in previous samples respectively.

Results exposed in Table 3 professed that 7 (70%) of Bellilah samples were positive to AFB1 in average of 0.216 ppb and 3 (30%) for AFM1. Regarding to rice milk were positive for AfB1 and AfM1 in 20 and 30% respectively.

Discussion

When food from animal origin and plant origin are mixed together in a planned manner, we will undoubtedly get a new high nutritional value product. Dairy desserts developed by mixing milk with different cereals are considered as such product. It also gives a taste that is wonderful and distinct and satisfies all tastes. Even to those who do not like or could not consume liquid milk, it is possible that the variations of tasteful dairy products are the solution.

But although cereals are a rich source of macro and micro nutrients, they may also be a source of more contamination

Table 1. Total mold count/ gram of commercial and homemade dairy desserts samples

Samples	Examined samples	Positive samples Day 1		CFU/ g	Positive samples Day 3		CFU/g
		No.	%	Mean ± SD	No.	%	Mean ± SD
Commercial							
Bellilah	30	16	53.3	$9.33x10^2±0.82x10^2$	22	73.3	$2.8x10^3±0.123x10^3$
Rice Milk	30	5	16.6	$2.9x10^2±0.14x10^2$	13	43.3	$8.0x10^2±0.91x10^2$
Muhallabiah	30	21	70	$11.9x10^2±3.31x10^2$	25	100	$4.7x10^3±0.129x10^3$
Control Homemade							
Bellilah	20	0	0	0	1	5	1 x 10
Rice Milk	20	0	0	0	0	0	0
Muhallabiah	20	0	0	0	2	10	1 x 10

Table 2. Mold genera isolated from dairy desserts

Mold spp.	Bellilah		Rice Milk		Muhallabiah	
	No	%	No	%	No	%
A. flavus	6	16.2	1	7.6	12	21.4
A. niger	3	8.1	1	7.6	12	21.4
A. ochraceous	0	0	1	7.6	2	3.5
A. nomius	0	0	0	0	1	1.7
Acremonium spp.	0	0	0	0	2	3.5
Alternaria	6	16.2	2	15.3	17	30.3
Cladosporium spp.	2	5.4	2	15.3	3	5.3
Fusarium spp.	0	0	1	7.6	1	1.7
Mucor spp.	6	16.2	0	0	4	7.1
P. species	14	37.8	5	38.5	2	3.5
Total	37	100	13	100	56	100

Table 3. Concentrations of Aflatoxins B1 and M1 in dairy desserts

	Examined samples	AFB1 concentration (ppb)			No. of positive samples	No. of samples above ES	AFM1 concentration (ppb)			No. of positive samples	No. of samples above ES	No. of samples above EUTL
		Min.	Max.	Mean ± SD			Min.	Max.	Mean ± SD			
Commercial												
Bellilah	10	0.088	0.85	0.216±0.188	7	7 (70%)	0.001	0.250	0.097±0.033	3	3 (30%)	1 (10%)
Rice Milk	10	0.05	0.09	0.07±0.028	2	2 (20%)	0.022	0.125	0.061±0.055	3	3 (30%)	1 (10%)
Muhallabiah	10	0.052	0.37	0.168±0.136	6	6 (60%)	0.013	0.156	0.052±0.069	4	4 (40%)	1 (10%)
Control Homemade												
Bellilah	10		0.057		1	1(10%)		-		-	-	-
Rice Milk	10		-		-	-		-		-	-	-
Muhallabiah	10		-		-	-		-		-	-	-

ES (Egyptian Standards): Dairy products must be free from aflatoxins.

EUTL (European Union Tolerance Limits) for AFM1 is 0.05 ppb. (European Commission, 2006).

by the spores of xerophilic molds that prefers dryness, especially with poor storage conditions. (Alim *et al*., 2018).

In this study, the mycological analysis of 90 commercial dairy desserts declared that high levels of mold count compared to the controlled homemade samples (n=60) in both day 0 of processing and after 3 days of cold storage as shown in Table 1.

On day 0, the commercial Bellilah and Muhallabiah samples have the highest incidence of contaminated samples 53.3 and 70%, respectively and highest mold count per g, ranged around $9.33x10^2$ and $11.9x10^2$ CFU/g, while, homemade (HM) samples have shown no mold growth at the first day of production, this is due to application of good manufacturing conditions and using of good quality heat treated milk and cereals, and clean utensils and hands.

After 3 days of cold refrigeration at 4°C, the test was repeated and a huge increase in TMC in Muhallabia average of $4.7x10^3$/g was recorded, this may be due to Muhallabia is more perishable than other desserts and cooked by a temperature lower than that required for Bellilah and Rice Milk. As well as molds are xerophilic, psychrotolerant and can growing, spoil sweeten refrigerated dairy desserts.

Fungal contamination of Muhallabia with high incidence gives an indication of improper sanitation and neglected hygienic measures after preparation, during distribution in containers or during holding till serving.

On the other hand, after 3 days the HM samples were still fresh with normal color and odor and comply with Egyptian standards (2003). These findings go in accordance with those of Montage Montagna *et al*. (2004); Chowdhury *et al*. (2011); Berikten and Kivanc (2012) and Khalifa *et al*. (2013). On the other side, a high incidence was obtained by Al-Gendi (2004).

Table 2 showed the isolated mold species from different desserts. Among 37 isolates in Bellilah, *Penicillium* have the predominance (37.8%) and *Aspergillus* spp was the most frequent in Muhallabiah, this may be due to wheat grains (in Bellilah structure) are very sensitive and spoiled rapidly. While, rice showed some resistance to mold growth. Relatively similar results were recorded by Moubasher *et al*. (2018), whom used DG18 medium to isolate filamentous fungi from dairy products in Egypt and recovered genera of *Aspergillus*, Cladosporium and *Penicillium* from all types of samples.

Garnier *et al*. (2017) can identify 10% *Penicillium* spp. from total of 41 isolates of molds from spoiled dairy foods and dairy environment. Also *Cladosporium phyllophilum* and *Cl. halotolerans* were isolated.

Aspergillus niger and *Alternaria* appeared heavily on Muhallabiah samples during storage due to poor sanitation and hygiene during processing, which allows contamination by mold spores. Sakkas *et al*. (2014) and Khalifa *et al*. (2013) obtained similar results.

Table 3, confirmed concentrations of AFLB1 in commercial and HM samples. It is obvious that Bellilah and Muhallabiah commercial samples showed the highest number of positive AFB1 samples as well as highest concentrations exceeding permissible limits of Egyptian standards (free) and European Committee Regulations (2 ppb for AFB1). The commercial Bellilah samples had shown 70% of contaminated samples over Egyptian standards (ES), 2003 permissible level in average mean of 0.216 ppb, however 60% of Muhallabiah samples (mean 0.168ppb) were above these limits which mean great health hazard to the consumer, especially children.

Alim *et al*. (2018) evaluated levels of mycotoxins in 229 samples of different cereals and cereal products and revealed that 53% of samples were contaminated by AFB1. from examined wheat samples, 67 % were recorded as positive for AFB1, Corn samples were contaminated by AFB1 in percentage of 44 and mean of 1.80 ppb. While, 50% of corn flour samples were positive for AFB1.

This finding confirms manufacturing of commercial Bellilah from low-quality wheat grains stored under high relative humidity and became contaminated with mold and its toxins, which pass to end product without being affected by processing. On the other hand, using of good quality wheat in HM Bellilah samples revealed a product of AFB1 lower than ES permissible limits.

Aflatoxin M1 is the derivative of AFB1, which metabolized by the liver to hydroxylated form AFM1 that secreted with milk. So it is less toxic than AFB1 but it is still toxic and considered of great human health hazard especially with frequent use.

Many researchers detected levels of AFM1 in different dairy and dairy based products all over the world as Ertas *et al*. (2011) in Turkey; Campone *et al*. (2015) in Italy; Khalifa *et al*. (2013) and Elgerbi *et al*. (2014) in Egypt and North Africa; Shuib *et al*. (2017); Hassan *et al*. (2018) in Jordan and Qatar and Campagnollo *et al*. (2016) in Brazil.

They found very different levels and percentages of aflatoxin M1 in dairy products. This wide difference is due to several reasons, such as climate variability, food storage conditions, total aflatoxins levels in various animal feeds, dairy products processing methods and others.

Table 3 showed that all the three types of examined commercial desserts were contaminated with AFM1 in a nearly equal manner (30:40 %) in levels ranged between 0.001to 0.250 ppb. Relatively higher percentages of positive samples were reported in dairy desserts by Mutlu *et al*. (2010) and Xiong *et al*. (2018) 54 and 65 and 73.6% respectively.

Ertas *et al*. (2011) found AFM1 in 26 (52%) out of 50 dairy desserts samples in Turkey by ELIZA technique in levels ranging from 1.5 to 80 ng/kg.

In this study, all positive samples were exceeded the ES and about 10% of each dessert type exceed EC limits (0.05 ppb for AFM1) similar to Hussain *et al*. (2008), who reported 13.9%.

On the other hand, 30 (100%) of HM samples were free from AFM1. These findings confirms the processing of commercial dairy desserts by using of low-quality milk contaminates with AFM1 came from dairy animals ingested contaminated rations with aflatoxins. (Martínez and Blasco 2015; Ogunade *et al*., 2017) conveyed a connection between amount of total aflatoxins in cow feed and AFM in produced milk.

Like other countries, Egypt has problems regarding the polluted environment and bad sanitation, which have a negative and direct impact on health. Consequently, dairy desserts and their ingredients may be contaminated with various molds. Unfortunately, the hygienic measures followed may be under way in most due to the absence of adequate hygiene and regular mycological control, as well as, lacking of standards and specifications for methods of manufacturing practices and examination. Moreover, the data dealt with the quality for dairy desserts are sketchy or totally absent.

Conclusion

Dairy desserts are so popular to the consumer but of short shelf-life. So that manufactured in small scales levels. Due to additives and changes of its consistency and appearance than original milk, it is easy to be adulterated by using of poor quality milk and/or cereals depending on the processing and additives will cover any defect so the consumer is subjected to a biological hazard. For that, more hygienic measures especially General Manufacturing Practice must be applied on small scales restaurants of dairy desserts in Egypt. On the other hand, controlled homemade samples were of great quality and long shelf-life due to using of good quality ingredients and equipment.

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