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The Role of Probiotic Bacteria in Protecting against Aflatoxin M1 Contamination in Milk and Certain Dairy Products

Rabie, M. A.[1]*; E. M. Abd El-Wahed[1]; M. G. Moustafa[2]; Kh. El-Zahar[1,3] and A. M. Abdel-Zaher[4]
[1] Department of Food Science, Faculty of Agriculture, Zagazig University, Zagazig, E- 44519 Egypt
[2] Department of Microbiology, Faculty of Agriculture, Zagazig University, 44517 Zagazig, Egypt
[3]Department of Food Science and Human Nutrition, Qassim University, Buraydah, Qassim, Saudi Arabia
[4]Chemistry department, Faculty of Science, Zagazig University, 44551 Zagazig, Egypt
***Corresponding author: Mohamed A. Rabie, PhD**
E-mail address: Marabie@agri.zu.edu.eg; Phone number: +2-055-2379224;
Fax number: +2-055-2287567

ABSTRACT

Purpose:The present study investigated AFM_1 contamination in raw milk, kareish cheese and yoghurt and studied the abilities of heating and probiotic bacteria to reduce AFM_1 contamination in both raw milk and yoghurt. Methods: AFM_1 was detected in raw milk in 10 of 12 samples (83.3%) from each season. Two methods were used to assess the toxigenic potential. HPTLC was performed for aflatoxin detection. Aflatoxin M_1 contamination milk and selected dairy products was investigated against heating and *Lactobacillus acidophilus* La_5 and *Bifidobacterium lactis* Bb_{12} and its combination. Result: Cheese had the highest mean values concentration of AFM_1 (527.4 and 651.3 ng/kg), and yoghurt had the lowest concentration (39.13 and 64.68 ng/kg) while, raw milk samples were (207.0 and 311.8 ng/kg) during summer and winter, respectively. The concentration of toxin in 83.33% of the examined cheese samples exceeded the EU limit (50 ng/kg), and 70.83% of these samples exceeded the Codex limit (500 ng/kg) during both seasons. Boiling degraded 26.71% of the AFM_1. Whereas, pasteurization only degraded 15.45%. In yoghurt, two strains of probiotic bacteria (*Lactobacillus acidophilus* and *Bifidobacterium lactis*) each gradually reduced the AFM_1 concentration as a function of time in milk contaminated with 25 ng/l, with complete elimination by the end of the refrigerated storage period (3 days), while, the combination of both probiotic bacteria (*L. acidophilus* and *B. lactis*) was better able to reduce AFM_1 in milk contaminated with 50.0 ng/kg or 75 ng/kg AFM_1.Conclusion: The most extensive reductions of the AFM_1 concentration were to 41.80 ng/kg (45.3%), 22.6 ng/kg (69.90%) and 7.12 ng/kg (92.8%), which were achieved using the same concentration of each strain individually and in combination, respectively, after two days in milk contaminated with 75 ng/kg. No AFM_1 was detected after three days using the combined strains.

Keywords: Aflatoxin M_1, raw milk, milk products, heating, probiotic bacteria.

INTRODUCTION

Aflatoxins are a group of secondary toxic and carcino genic metabolites produced by different species of Aspergillu s such as *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* (Ito *et al*. 2001). The growth of *Aspergillus* toxic species on dairy product may result in one or more aflatoxins contaminating that product. If made from contaminated milk, cheese can possibly contain aflatoxin M_1 (AFM_1), as well as B_1 and other aflatoxins if the cheese subsequently supports the growth of toxigenic *Aspergilli* (van Egmond and Dragacci 2001). Regulation (EC) 1881/2006 and subsequent amendments set maximum levels for mycotoxins and certain other contaminants in food.

Regulation (EC) 401/2006 introduced provisions of sampling and analysis methods for the official control of mycotoxins RASFF Regulation Commission regulation (EU) No 16/2011, FDA (2011); USDA (2016).

AFM_1 is the hydroxylated metabolite of aflatoxin B_1 produced by the action of the cytochrome P450 oxidase system present in the ruminal microflora and cells of animals and can be found in milk and later on in other dairy products when lactating animals are fed with contaminated feedstuffs (van Egmond *et al*. 2007; Motawee *et al*. 2009); (Dashti *et al*. 2009). (Bakirci 2001) detected varying increases in the AFM_1 content of yoghurt in relation to contaminated milk. Fermentation effect was assessed by (Govaris *et al*. 2002) who found that levels of AFM_1 were significantly lower for all yogurt samples than those initially present in milk. Factor such as low pH, the formation of organic acids and other by-products of fermentation, and the presence of lactic acid bacteria (LAB) were attributed to this reduction of AFM_1. Contamination levels of AFM_1 were significantly higher in autumn and winter samples than in spring and summer samples (Kamkar, 2005).

The European Union (No 2006) established lower maximum allowable levels for AFM_1 at 50 ng/kg for milk and 250 ng/kg for cheese. The European Union standards have been followed in many other countries (Dashti *et al*. 2009). In Egypt, the ministry of health established in 1990 that fluid milk and dairy products should be free of AFM_1, and the current maximum permissible levels follow the European Union standard (Egyptian Standard 1990). AFM_1 in milk products represents a serious risk to health hazard of consumers, especially kids, who are more sensitive than adults to aflatoxin's adverse effects (Fallah 2010).

A total of 50 raw milk samples and 150 from samples were analysed (each of 50 from soft cheese, hard cheese, and processed) and the mean concentrations of AFM_1 were found in the soft and hard cheese samples above those in the raw milk samples ; the cheese processing samples were the least contaminated (Amer and Ibrahim 2010). All positive raw milk and cheese samples have exceeded Egyptian regulation (free from AFM_1), whereas they are all subject to US regulation (500 ng/l or kg) and have exceeded European Commission regulations on all positive cheese samples and 52.6 % of the raw milk samples examined (50 ng/l or kg).

Motawee (2013) studied the elevated levels of AFM_1 in milk and milk products and found that the conversion of milk into Domiati cheese and the subsequent three-month storage period of this cheese reduced AFM_1 levels overall by 64%.

AFM_1 can be detected in milk 12–24 h after the initial aflatoxin B1 ingestion, then AFM_1 concentration in the milk decrease to an undetectable level within 72 h (Rahimi and Karim, 2008). Temperature and moisture were the most important factors in the amount of aflatoxin, as molds like Aspergillus flavus and Aspegillus parasititicus can grow easily in feed with moisture from 13% to 60% and environmental moisture from 50% to 60%. (Kamkar *et al*. 2014). Furthermore, AFM_1 pollution levels in dairy products may be attributable to the fact that forage and compound-stored feeds used during cold seasons are higher in APB1

compared to feeds used during warm seasons, including fresh grass (Kamkar *et al*. 2014). The ratio of excreted AFB_1 has been estimated to be 1–3% (Fallah 2010). AFM_1 is also very stable and is not destroyed through storage or processing such as pasteurization, autoclaving or other techniques used to manufacture fluid milk and AFM_1 could persist with the final products for human consumption if present in raw milk (Tajkarimi *et al*. 2008). Bakirci, (2001) reported that sterilizing milk at 121°C for 15 min degraded AFM_1 by 12.21% and that boiling at 100 °C for 20 min decreased AFM_1 by 14.50%, the most efficient removal of AFB1 was achieved by heat-killed bacteria and concluded that destruction of AFM_1 depends on time and temperature combination of the heat treatment applied. Fresh cow's milk at 121 °C sterilization resulted in a significant AFM_1 decrease ($p<0.05$), of up to 58.8 % (Omeiza *et al*., 2018).

The thermal bacteria can remove AFB1 as viable as bacteria, therefore a possible mechanism for metabolic degradation through viable bacteria in such experiments has been excluded. In this stage, the disappearance of aflatoxin could be due to binding of aflatoxin to the bacterial cell wall, a mechanism that other reports also suggested.

Recently, El-kest *et al*. (2015) highlighted the serious risk to public health since all age groups, including infants and children, consume milk and milk products worldwide. Milk and milk products therefore need to be monitored continuously for contamination with AFM_1. It is also extremely important to maintain low levels of AFB1 in milk feed. (Bakirci 2001) reported that sterilizing milk at 121°C for 15 min degraded AFM_1 by 12.21% and that boiling decreased AFM_1 by 14.50%, this destroying of AFM_1 depends on the time and temperature combination of the heat treatment applied. Seasonal effects influence concentrations of AFM_1, many authors have shown. The concentration of AFM_1 is reported in cold seasons to be higher than in hot seasons, as in winter lactating cow food is fed by more mixed feeds that could be contaminated at higher AFB1 levels (Hussain and Anwar 2008; Tajkarimi *et al*. 2008; Bilandžić *et al*. 2010; Fallah 2010).

Many probiotic and LAB strains originated in fermented foods have been shown to inhibit both mould growth and mycotoxin production (ElShafei *et al*. 2010).

The present study investigated AFM_1 contamination in raw milk, kareish cheese and yoghurt and studied the abilities of heating and probiotic bacteria to reduce AFM_1 contamination in both raw milk and yoghurt.

MATERIALS AND METHODS

Mould Isolation

A 0.1ml aliquot of each sample dilution (milk, kareish cheese and yoghurt) was spread onto the surface of solidified Martin's medium Baruah and Barthakur (1997) and yeast extract-glucose-chloramphenicol-blue agar medium (YGCB agar). The petri dishes were incubated at 30±2°C for 5 days, and the colonies were then counted. Single mould colonies were removed, streaked on YM agar slants in test tubes, and stored at 4°C until use.

Evaluation of the Toxigenic Potential of the Isolated Strains

Two methods were used to assess the toxigenic potential: a medium-based qualitative system and high-performance liquid chromatography. Analyses for quantifying aflatoxin production were performed as follows:

Qualitative assay

This assay was performed according to the method of Dyer and McCammon (1994). In the case of an immediately prepared coconut agar, isolates of the Aspergillus flavus and related species have been developed for the detection of aflatoxin production. A. flavus isolates were detected more effectively than the synthetic medium, which included coconut cream (50 %) and agar (1.5 %), and were just as efficient as media containing desiccated coconut. Colonies produced on coconut cream agar were differentiated by fluorescence coloring to A. flavus from A. parasiticus and A. nomius. Furthermore, conidial colour of A. flavus and A. nomius was very different from that of A. parasiticus.

Quantitative analysis of aflatoxin

HPTLC was performed according to the method of Yuanling *et al*., (1996). HPTLC Separation Procedures. Chromatographic separations were performed on either 5 cm × 10 cm or 10 cm ×10 cm HPTLC plates coated with silica gel 60 (E. Merck, Darmstadt, FRG). All plates were chromatographically washed three times with methanol and allowed to dry at room temperature prior to sample application. The plate prewash procedure was performed in order to reduce the fluorescence background from any adsorbed organic impurities on the plate. Quantitative TLC. Aliquots of 0.5 µL of sample extracts were spotted onto a 5 cm × 10 cm HPTLC plate adjacent to aliquots of standards ranging from 5 to 125 pg for M1 per spot. The plate was then developed with anhydrous ethyl ether to remove the interfering compounds (direction 1) until the solvent front reached the top of the plate. The ethyl ether was evaporated, and the plate was examined by the CCD camera system. The top portion of the silica sorbent layer containing the interfering compounds, approximately 1 cm, was scratched off. To separate the aflatoxin species, the plate was turned 180° and developed with chloroform/acetone (9:1 v/v) or chloroform/ethyl ether (7:3 v/v) until the solvent front migrated to a distance of 7.5 cm. The chromatographic images were obtained after the developed plate was dried in air for 5 min.

Effect of Heating on Aflatoxins

Milk sample inoculation

AFM_1-negative milk samples (total volume of approximately 10 l) were mixed, divided into 4 groups, and inoculated with 10, 5, 2.5 or 1.25 ng/kg AFM_1 standard.

Treatment of inoculated samples

Twenty four samples of each group (raw milk, kareish cheese and yogurt) were used for aflatoxin detection in winter and summer season. Each group was divided into 3 subgroups of investigated samples (100 ml or g to each). The 1st subgroup served as the control. The second subgroup was pasteurized at 65°C for 30 min, followed by sudden cooling at 4°C. The third subgroup was boiled at 100°C for 10 min (Shils, 1994).

AFM_1 Detoxification in Yoghurt by LAB

Culture activation

LAB were obtained from the Christian Hansen (Chr. Hansen A/S laboratory, Copenhagen, Denmark). The cultures were activated in 11% reconstituted skim milk several times, and the last 3 activations were in strain-specific medium at 37°C.

LAB inoculum preparation

Lactobacillus acidophilus La_5 and *Bifidobacterium lactis* Bb_{12} were cultivated in 25 ml of De Man Rogosa and

Sharpe (MRS) broth and agar (Oxoid CM 359) at 37°C for 24 h (Oxoid manual, 1998). *B. lactis* was cultivated in 25 ml of MRS broth (Oxoid 358) at 37°C for 24 h. The suspensions were centrifuged at 1700×g for 15 min under cooling (10°C). The bacterial pellets were washed with phosphate saline buffered (PBS; pH 7.3, 0.01 M) twice and the supernatants were removed. The LAB and *Bifidobacterium* were counted using traditional plate counting and were adjusted to 3×10^8 and 7.6×10^6 cfu/ml bacteria per 4 ml of PBS (per tube), respectively.

Binding ability of LAB to AFM_1

To study the binding ability of LAB, *L. acidophilus* La_5 (2%) and *B. lactis* Bb_{12} (2%) were combined. A millilitre of a combination of *L. acidophilus* La_5 (1%) and *B. lactis* Bb_{12} 1% (0.5 ml of each) was suspended in separate Falcon tubes containing 49 ml of commercial ultra-high temperature (UHT) skim milk naturally contaminated with AFM_1 concentrations of 25, 50 and 75 ng/kg and incubated at 37°C for 5 h. The unbound content of AFM_1 was determined by HPTLC analysis after 24, 48 and 72 h in storage at 4±1°C according to AOAC (2005). The toxin was measured using HPTLC, and cell-free milk contaminated with aflatoxin was used as a positive control. Non-contaminated skim milk suspended bacteria have been used as a negative control (pure species), and all testing has taken place in triplicate (Mohamed 1998; Elsanhoty *et al*., 2014)

Statistical analysis

The experiments were conducted in triplicate and were statistically analyzed on triplicate samples using a computer program "SAS system for windows version 9.00 TS M0" (SAS 2008) for analysis of variance by one way (ANOVA) and comparison of means by Duncan's multiple comparison test where $P < 0.05$ was considered for significant difference

RESULTS AND DISCUSSION

Mould contamination not only deteriorates food and animal feed but also adversely affects human health. Moreover, fungi influence the biochemical character and flavour of the product, which often downgrades the product.

Table 1 shows the results from 72 milk and dairy product samples (cheese and yoghurt) tested for moulds that produce AFM_1. Moulds producing AFM_1 were found in 70.83% (17 of 24) of the raw milk samples. However, 91.67% (22 of 24) Kareish cheese samples were positive, and 12 of 24 yoghurt samples (50%) were positive.

Table 1. Incidence of moulds producing aflatoxin M_1 in dairy and dairy products.

Examined samples	Total No. of examined samples	Moulds			
		Positive		Negative	
		No.	(%)	No.	(%)
Raw milk	24	17	70.83	7	29.17
Kareish cheese	24	22	91.67	2	8.33
Yoghurt	24	12	50.00	12	50.00

Aflatoxin in Raw Milk

The data presented in Table 2 shows AFM_1 occurrence and its levels (ng/kg) in both the summer and winter seasons of raw milk. AFM_1 was detected in 10 of 12 samples from each season. AFM_1 concentrations were higher during the winter season than during the summer, with average values of 311.8 and 207.0 ng/kg during the winter and summer, respectively.

These results are higher than those recommended by the European Union (European Commission, 2006). The standards of the European Union were followed by many other countries (Dashti *et al*. 2009). In Egypt 1990, the Ministry of Health set up raw milk and dairy products free of AFM_1, and recently the maximum permissible levels comply with the EU standard. An increase of AFM_1 in milk products and dairy products exceeding Codex limit may therefore affect international trade in these dairy products on global markets for any country, including Egypt.

The present results revealed that AFM_1 concentrations in milk samples were higher in the winter than in the summer, which is consistent with the results of Tajkarimi *et al*. (2008), Who reported significantly higher levels of AFM_1 in winter milk samples than in summer ($P<0.05$); 30% of winter samples were >50 ng/kg and only 16% of summer samples were >50 ng/kg. One reason for this result is that milking animals are fed with greater amounts of mixed feed that may be contaminated at higher levels of AFB1 in winter season (Kamkar 2005; Hussain and Anwar 2008).

Table 2. Occurrence of aflatoxin M_1 in raw milk and its concentration (ng/kg) in summer and winter seasons.

Summer season	Milk samples	AFM_1 (ng/kg)	Winter season	AFM_1 (ng/kg)
June 2015	1	$9.80^{h}\pm0.24$	December 2016	$000.00^{k}\pm0.00$
	2	$86.2^{f}\pm0.55$		$100.90^{i}\pm0.50$
	3	$538.0^{a}\pm0.92$		$520.43^{c}\pm0.44$
	4	$422.0^{b}\pm1.60$		$386.31^{f}\pm0.49$
July 2015	1	$0.00^{i}\pm0.00$	January 2017	$78.600^{j}\pm0.33$
	2	$66.73^{g}\pm1.04$		$246.05^{h}\pm0.78$
	3	$423.01^{b}\pm0.42$		$577.80^{b}\pm0.90$
	4	$309.4^{d}\pm0.57$		$392.40^{e}\pm1.33$
August 2015	1	$0.00^{i}\pm0.00$	February 2016	$00.000^{k}\pm0.00$
	2	$8.30^{h}\pm0.33$		$274.02^{g}\pm0.07$
	3	$339.5^{c}\pm1.59$		$698.30^{a}\pm0.57$
	4	$291.1^{e}\pm0.49$		$467.12^{d}\pm0.51$
Mean		207		311.8
LSD 0.05		1.08		1.64
CV%		0.20		0.47
F test		**		**
P value		0.000		0.000

From 1-4 of each season are different milk sample collected from traditional market in Egypt

***Data were expressed by means ±SD (n=24); Values in the same column with different letters were significantly different according to Duncan's test ($p< 0.05$).**

Aflatoxin in Kareish cheese

The data presented in Table 3 show the occurrence and concentration (ng/kg) of AFM_1 in kareish cheese in both summer and winter seasons. AFM_1 was detected in all the samples (24) obtained during both seasons. The highest values of AFM_1 were 1295.0 and 1612.0 ng/kg, and the lowest values were 32.56 and 36.57 ng/kg in the summer and winter, respectively. Clearly, the concentrations of AFM_1 were lower in the summer than in the winter with average values of 527.4 versus 651.4 ng/kg.

The present study revealed that 83.33% of the examined Kareish cheese samples (20 of 24) that exceeded the EU limits (50 ng/kg) and 70.83% of the examined cheese samples (17 of 24 samples) exceeded the Codex limits (500 ng/kg) in both the summer and winter. In Egypt in 1990, the ministry of health established that fluid milk and dairy products should be AFM_1-free, and the current maximum permissible levels follow the European Union standard.

A later study was done by (Amer and Ibrahim 2010) who investigate 150 cheese samples (fifty each of soft cheese, hard cheese and processed cheese) and found the average concentrations of AFM_1 were higher in soft and hard cheese samples than in raw milk samples, while processed cheese samples were the less contaminated samples. All the positive cheese samples exceeded Egyptian regulations (free from AFM_1), while all were within US regulations (500 ng/kg), and the EU Commission's regulations (50 ng/kg) exceeded all positive cheese samples and 52.6% of crude milk samples. Another study in Egypt found that the AFM_1 content in kareish cheese samples ranged from 5000 to 35,000 ng/kg, with a mean value of 17,500 ng/kg (El-Diasty and Salem 2007). In addition, soft cheese (fresh kareish and Domiati) samples have been examined, and the mean values were 3600 and 67,000 ng/kg, respectively (Awad *et al.* 2014), while other researchers detected no aflatoxins in some cheese samples (Sessou *et al.* 2013; Fontaine *et al.* 2015).

Table 3. Occurrence of aflatoxin M_1 in kareish cheese and its concentration (ng/kg) in summer and winter seasons.

Summer season	Kareish cheese samples	AFM1 (ng/kg)	Winter season	AFM1 (ng/kg)
June 2016	1	$487.20^{f}\pm0.57$	December 2016	$36.570^{l}\pm0.35$
	2	$591.30^{d}\pm0.49$		$239.15^{i}\pm0.69$
	3	$1295.0^{a}\pm1.59$		$588.00^{e}\pm1.63$
	4	$762.45^{c}\pm0.49$		$467.04^{g}\pm0.85$
July 2016	1	$46.11^{k}\pm0.74$	January 2017	$184.36^{k}\pm0.56$
	2	$392.25^{i}\pm0.61$		$326.35^{h}\pm1.37$
	3	$856.09^{b}\pm0.51$		$1612.0^{a}\pm2.41$
	4	$458.00^{g}\pm1.61$		$755.09^{d}\pm0.44$
August 2016	1	$32.56^{l}\pm0.35$	February 2017	$227.64^{j}\pm0.29$
	2	$388.50^{j}\pm0.41$		$479.40^{f}\pm0.45$
	3	$566.70^{e}\pm0.42$		$1535.0^{b}\pm0.31$
	4	$453.65^{h}\pm1.08$		$1365.7^{c}\pm0.25$
Mean		527.4		651.4
LSD 0.05				
CV%				
F test		**		**
P value		0.000		0.000

From 1-4 of each season are different Kareish cheese sample collected from traditional market in Egypt,

***Data were expressed by means ±SD (n=24); Values in the same column with different letters were significantly different according to Duncan's test (p< 0.05).**

AFM_1 in Yoghurt

The data presented in Table 4 show the occurrence of AFM_1 in yoghurt and the AFM_1 concentrations (ng/kg) during both the summer and winter seasons. In the summer season, the highest AFM_1 concentration was 66.05 ng/kg, while the lowest concentration was 31.46 ng/kg, and AFM_1 was not detected in 3 of 12 samples. In the winter, the highest AFM_1 concentration was 84.14 ng/kg, while the lowest AFM_1 concentration was 56.60 ng/kg, and AFM_1 was not detected in 1 of 12 samples (Table 4).

Several surveys have been performed to determine AFM_1 levels in yoghurt. Approximately 80% of all yoghurt samples in Italy were contaminated with AFM_1, ranging from 1–3.1 ng/kg (Galvano *et al.* 1998). A further study found that 61.0 per cent of the yogurt samples had lower levels of AFM_1 than the previous survey.(Galvano *et al.* 2001). Forty eight yoghurt samples have been tested in Portugal, while only 2 (4.2 %) have been contaminated by 0.45 ng / kg AFM_1. Most yoghurt samples (62.88%) purchased at different markets in Ankara were free of AFM_1 Sarımehmetoglu *et al.* (2004), while elsewhere in Turkey, 65.38% of ordinary yoghurt samples, 33.33% of fruit yoghurt samples, and 55.77% of strained yoghurt samples contained aflatoxin (Akkaya *et al.* 2006). Another study found AFM_1 in 2.8% of yoghurt samples (Cano-Sancho *et al.* 2010). The levels of AFM_1 contamination in locally produced yoghurt appear to vary across studies. The different explanations for these variations include varying yoghurt manufacturing procedures, different milk contamination levels, yoghurt type, yoghurt ripening conditions, geographical region, the season and analytical methods employed (Guan *et al.* 2011).

Various AFM_1 content increases in yogurt associated with milk were detected (Bakirci 2001). Govaris *et al.*, (2002) evaluated fermentation effects and found that levels of AFM_1 have been significantly reduced in all samples of yogurt from those that were initially in the milk. Factors like low pH, organic acid formation and other fermentation by-products, and the presence of LAB, were attributable to this decrease in AFM_1. During fermentation the low pH alters dairy proteins structure, like caseins, leading to yogurt and cheese coagulum formation. These results were agreed with that obtained by Megalla and Hafez (1982) who concluded that the pH may contribute to the transformation of AFB1 to the non-toxic AFB2 in acidogenous yogurt. The fermentation of yoghurt and acidified milk containing AFB1 also significantly decreased the toxin level, accordingly (Rasic *et al*, 1991). The proportional link between both the degradation in pH values and the corresponding decline of aflatoxins, i.e. a further decrease in pH value and a further declines in the level of aflatoxins in Yogurt was established by (Motawee and Abd El-Ghany 2011).

Table 4. Occurrence of aflatoxin M_1 in yoghurt and its concentration (ng/kg) in summer and winter seasons

Summer season	Yoghurt samples	AFM1 (ng/kg)	Winter season	AFM1 (ng/kg)
June 2016	1	$00.00^{j}\pm0.00$	December 2016	$000.00^{l}\pm0.00$
	2	$55.07^{e}\pm0.35$		$56.60^{k}\pm0.24$
	3	$66.05^{a}\pm0.36$		$77.13^{d}\pm0.31$
	4	$56.89^{d}\pm0.33$		$65.50^{g}\pm0.33$
July 2016	1	$00.00^{j}\pm0.00$	January 2017	$62.96^{h}\pm0.29$
	2	$46.23^{g}\pm0.64$		$59.84^{j}\pm0.23$
	3	$62.06^{b}\pm0.42$		$84.14^{a}\pm0.22$
	4	$58.33^{c}\pm0.41$		$74.08^{f}\pm0.33$
August 2016	1	$00.00^{j}\pm0.00$	February 2017	$60.72^{i}\pm0.22$
	2	$31.46^{i}\pm0.44$		$75.39^{e}\pm0.40$
	3	$51.66^{f}\pm0.29$		$82.90^{b}\pm0.17$
	4	$41.85^{h}\pm0.26$		$79.85^{c}\pm0.56$
Mean		39.13		64.68
LSD 0.05				
CV%				
F test		**		**
P value		0.000		0.000

From 1-4 of each season are different Yoghurt sample collected from traditional market in Egypt

***Data were expressed by means ±SD (n=24); Values in the same column with different letters were significantly different according to Duncan's test (p< 0.05).**

Aflatoxin control

Two treatments were applied to reduce AFM_1 concentrations in raw milk and a milk product (yoghurt), including heating (boiling and pasteurization) and probiotic bacteria.

Effect of heating

Table 5 shows the mean concentration (ng/kg) and detoxification (%) of AFM_1 in different treated milk samples.

Clearly, applying heat reduced the AFM_1 concentrations in all the raw milk samples. These results revealed that pasteurization reduced AFM_1 concentrations in the raw milk samples by an average of 15.45%, ranging from 10.0 to 22.6%, while boiling reduced AFM_1 by an average of 26.7%, ranging from 25.47 to 28.57

Table 5. The mean concentration (ng/kg) and detoxification (%) of aflatoxin M_1 in different treated milk samples

Initial AFM1 levels (ng/kg)	Positive control levels (ng/kg)	Pasteurization treatments		Boiling treatments	
		Concentration (ng/kg)	Detoxification (%)	Concentration (ng/kg)	Detoxification (%)
Group I (10)	9.4a	8.00a	14.9	6.90a	26.60
Group II (5.00)	4.2b	3.60b	14.3	3.10b	26.19
Group III (2.50)	2.1c	1.89c	10.0	1.50c	28.57
Group IV (1.25)	1.06d	0.82d	22.6	0.79d	25.47
Average	4.19	3.58	15.45	3.07	26.71

*** Values in the same column with different letters were significantly different according to Duncan's test (p< 0.05).**

The present results are in good agreement with Choudhary *et al*. (1998), who reported that sterilizing milk at 121°C for 15 min caused a 12.21%, degradation of AFM_1, while boiling at 100 °C for 20 minutes decreased the concentration of AFM_1 destruction by 14.50 % according to a time and temperature thermal processing combination. A significant reduced level ($p<0.05$) of AFM_1 by up to 58.8% was achieved when fresh cow's milk was sterilized at 121°C for 15 min (Omeiza *et al*., 2018). Additionally, Bakirci, (2001) found that pasteurization decreased AFM_1 levels in milk at the rate of 7.62%. Also, UHT milk contamination levels were lower than those in raw milk. Based on the idea that heating or storing at lower temperatures does not appreciably change AFM_1 levels Prandini *et al*. (2009), many authors have shown that seasonal effects do influence AFM_1 concentrations.

Therefore, the heat treatment of milk may be critical for reducing AFM_1 levels and subsequently diminishing the dangers of this toxin to public health.

Effects of probiotic bacteria

Various species of primarily *Lactobacillus* and *Bifidobacterium* have historically been used as probiotics (Shahin 2007; Ranadheera *et al*. 2010).

Table 6 lists the results for two strains of LAB, *L. acidophilus* and *B. lactis* that were tested for AFM_1 reduction in milk contaminated with 25 ng/kgAFM_1. The level of toxin was clearly gradually reduced as a function of time, and the toxin was completely eliminated by the end of the refrigerated storage period (3 days), where *L. acidophilus* and *B. lactis* were better able to remove AFM_1. After one day, the AFM_1 concentration decreased to 17.8 ng/kg (28.8%), 10.4 ng/kg (58.4%) and 5.6 ng/kg (77.6%) in the presence of *L. acidophilus* (2%), *B. lactis* (2%) and the combination of *L. acidophilus* (1%) and *B. lactis* (1%), respectively. The most extensive reductions of the AFM_1 concentration to 8.6 ng/kg (65.6%), 6.9 ng/kg (72.4%) and 1.2 ng/kg (95.2%) were achieved using the same previous concentrations of LAB strains after 48 h. No AFM_1 was detected on the third day.

The two strains of LAB (*L. acidophilus* and *B. lactis*) were also tested for AFM_1 reduction in milk contaminated with 50 and 75 ng/kg AFM_1. The data indicate that after one day, the AFM_1 concentration (50 ng/kg) decreased to 37.5 ng/kg (25.0%), 24.4 ng/kg (51.2%) and 17.3 ng/kg (75.4%), while the concentration (75 ng/kg) was reduced to 62.7 ng/kg (16.4%), 51.5 ng/kg (31.3%) and 32.8 ng/kg (56.3%) in the presence of *L. acidophilus* (2%), *B. lactis* (2%) and the combination of *L. acidophilus* (1%) and *B. lactis* (1%), respectively. The most extensive reductions of the AFM_1 concentration (50 ng/kg) to 13.6 ng/kg (72.8%), 5.9 ng/kg (88.2%) and 2.6 ng/kg (94.4%), no AFM_1 was detected on the third day. While, the AFM_1 concentration (75 ng/kg) to 41.8 ng/kg (45.3%), 22.6 ng/kg (69.9%) and 7.12 ng/kg (92.8%) respectively were achieved using the same concentrations of LAB strains after 48 h. AFM_1 not detected in the treatment containing the combination of *L. acidophilus* (1%) and *B. lactis* (1%) on the third day; however, the AFM_1 concentration was reduced by *L. acidophilus* to 28.2 ng/kg (62.4%) and by *B. lactis* to 3.90 ng/kg (94.8%).

Table 6. Reduction of Aflatoxin M_1 (25, 50 and 75 ng/kg) in milk using *Lactobacillus acidophilus* (La_5) and *Bifidobacterium lactis* (Bb_{12})

Treatment	AFM_1 (ng/kg)	Control		*Lactobacillus acidophilus*		*Bifidobacterium lactis*		(La_5) + (Bb_{12})	
Days		Reduction of AFM_1	(%)	Reduction of AFM_1	(%)	Reduction of AFM_1	(%)	Reduction of AFM_1	(%)
1	25	22.7^{g}±0.18	9.2	17.8^{e}±0.08	28.8	10.4^{d}±0.08	58.4	5.6^{d}±0.09	77.6
2	25	12.2^{h}±0.15	51.2	8.6^{g}±0.13	65.6	6.9^{e}±0.13	72.4	1.2^{f}±0.04	95.2
3	25	7.8^{i}±0.11	68.8	ND^{n}±0.00	100	ND^{n}±0.00	100	ND^{g}±0.00	100
1	50	48.9^{d}±0.32	2.2	37.5^{c}±0.18	25.0	24.4^{b}±0.40	51.2	17.3^{b}±0.18	65.4
2	50	44.3^{e}±0.19	11.4	13.6^{f}±0.24	72.8	5.9^{f}±0.08	88.2	2.6^{e}±0.18	94.4
3	50	39.8^{f}±0.17	20.4	ND^{n}±0.00	100	ND^{n}±0.00	100	ND^{g}±0.00	100
1	75	72.4^{a}±0.22	3.5	62.7^{a}±0.16	16.4	51.5^{a}±0.19	31.3	32.8^{a}±0.20	56.3
2	75	70.1^{b}±0.25	6.5	41.8^{b}±0.12	45.3	22.6^{c}±0.16	69.9	7.12^{c}±0.22	92.8
3	75	68.6^{c}±0.16	8.5	28.2^{d}±0.13	62.4	3.90^{g}±0.10	94.8	ND^{g}±0.00	100
Mean		42.97		23.35		13.96		7.40	
LSD 0.05									
CV%				**					
F test		**		0.000		**		**	
P value		0.000				0.000		0.000	

ND: Not Detected

***Data were expressed by means ±SD (n=12); Values in the same column with different letters were significantly different according to Duncan's test (p< 0.05).**

Concerning the effect of LAB on reducing the concentration of AFM_1, these results are consistent with those reported by Mohamed, (1998), who measured reductions of AFM_1 in yogurt prepared with 95,3 % *L. acidophilus* , and 84,7 % *Bifidobacterium bifidum* after 5 days. The same conclusion was reached when different species of LAB were

used, and the reduction level by these strains ranged from 26.2–34.0% depending upon the bacterial isolates (Emara *et al*. 2000). Regarding AFM_1 stability in the cold, El Khoury *et al*. (2011) found that the LAB (*L. bulgaricus* and *S. thermophilus* strains) used in Lebanese dairy industries effectively reduced AFM_1 concentration in liquid culture media and during yoghurt processing. LAB thus seems to play a key part in removing AFM_1 and could be employed in the reduction of AFM_1 levels as a biological agents. Results obtained in this study showed that all of the LABs and *bifidobacteria* under this study are capable to bind AFM_1.

CONCLUSION

Raw milk, kareish cheese and yoghurt were assessed for their contamination with aflatoxin (AFM_1). The occurrence and concentrations of AFM1 varied with product type and season of the year. The concentration of toxin in 83.33% of the examined cheese samples were exceeded the EU limit (50 ng/kg) and 70.83% of these samples exceeded the Codex limit (500 ng/kg) during both seasons. The combination of both probiotic bacteria (*L. acidophilus* and *B. lactis*) was better able to reduce AFM_1 in milk by 78% after one day of incubation. It could be concluded that the effect of both heat treatment and probiotics differed with the initial level of AFM_1 concentration.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Research involving human participants and/or animals

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Informed consent

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REFERENCES

Akkaya L, Birdane YO, Oguz H, Cemek M (2006) Occurrence of aflatoxin M~ 1 in yogurt samples from Afyonkarahisar, Turkey Bulletin-Veterinary Institute in Pulawy 50:517

Amer AA, Ibrahim ME (2010) Determination of aflatoxin M1 in raw milk and traditional cheeses retailed in Egyptian markets Journal of Toxicology and Environmental Health Sciences 2:50-52

AOAC Official Method 2000.08. (2005). Natural toxins-chapter 49 (18th ed.). In Official Methods of Analysis of AOAC InternationalAflatoxin M1 in liquid milk, immunoaffinity column by liquid chromatography (pp. 45e47). Gaithersburg, Maryland 20877-2417, USA: AOAC International.

Awad EI, Mansour IHAH, Ismail S-EY (2014) Qualitative and Quantitative Detection of Aflatoxin M1 Residues in White Soft Cheese Alexandria Journal of Veterinary Sciences 40:119-123

Bakirci I (2001) A study on the occurrence of aflatoxin M1 in milk and milk products produced in Van province of Turkey Food control 12:47-51

Baruah T, Barthakur H (1997) A Textook of Soil Chemical Analysis Vikash, New Delhi

Bilandžić N, Varenina I, Solomun B (2010) Aflatoxin M 1 in raw milk in Croatia Food Control 21:1279-1281

Cano-Sancho G, Marin S, Ramos AJ, Peris-Vicente J, Sanchis V (2010) Occurrence of aflatoxin M1 and exposure assessment in Catalonia (Spain) Revista Iberoamericana de Micología 27:130-135

Choudhary P, Sharma R, Borkhateria V (1998) Carry-over of aflatoxin B1 from feed as aflatoxin M1 in milk of Indian cows Milchwissenschaft 53:513-515

Dashti B, Al-Hamli S, Alomirah H, Al-Zenki S, Abbas AB, Sawaya W (2009) Levels of aflatoxin M1 in milk, cheese consumed in Kuwait and occurrence of total aflatoxin in local and imported animal feed Food Control 20:686-690

Dyer SK, McCammon S (1994) Detection of toxigenic isolates of Aspergillus flavus and related species on coconut cream agar Journal of Applied Bacteriology 76:75-78

Egyptian Regulations. 1990. Maximum Limits for Mycotoxin in Foods. Part L Aflatoxins E.S.1875-1990

El-Diasty EM, Salem R (2007) Incidence of lipolytic and proteolytic fungi in some milk products and their public health significance J Appl Sci Res 3:1684-1688

El-kest MM, El-Hariri M, Khafaga N, Refai MK (2015) Studies on Contamination of Dairy Products by Aflatoxin M1 and Its Control by Probiotics J Glob Biosci 4:1294-1312

El Khoury A, Atoui A, Yaghi J (2011) Analysis of aflatoxin M1 in milk and yogurt and AFM1 reduction by lactic acid bacteria used in Lebanese industry Food Control 22:1695-1699

Elsanhoty, RM, Salam, SA, Ramadan, MF and Badr FH (2014) Detoxification of aflatoxin M1 in yoghurt using probiotics and lactic acid bacteria Food Control 43: 129-134

ElShafei GM, El-Said M, Attia HA, Mohammed T (2010) Environmentally friendly pesticides: Essential oil-based w/o/w multiple emulsions for anti-fungal formulations Industrial crops and products 31:99-106

Emara H, Bean G, Trucksess M (2000) Detoxification of aflatoxin M1 on lactic acid bacteria in contaminated with it milk J Agric Sci Mansoura Univ Egypt 25:945-953

European Commission. (2006). Commission Regulation (EC) No. 1881/2006 of 19 December 2006-setting maximum levels for certain contaminants in foodstuffs. Regulation (EC) 401/2006 on the official control of the levels of mycotoxins in foodstuffs ttp://eurlex.europa.eu/ Lex UriServ / Lex Uri Serv. do? uri0 CONSLEG: 2006 R1881: 20100701: PT:PDF Accessed 10.08.13.

Fallah AA (2010) Aflatoxin M 1 contamination in dairy products marketed in Iran during winter and summer Food control 21:1478-1481

Fontaine K *et al*. (2015) Occurrence of roquefortine C, mycophenolic acid and aflatoxin M1 mycotoxins in blue-veined cheeses Food Control 47:634-640

Galvano F, Galofaro V, DE ANGELIS A, Galvano M, Bognanno M, Galvano G (1998) Survey of the occurrence of aflatoxin M1 in dairy products marketed in Italy Journal of Food Protection 61:738-741

Galvano F, Galofaro V, Ritieni A, Bognanno M, De Angelis A, Galvano G (2001) Survey of the occurrence of aflatoxin M1 in dairy products marketed in Italy: second year of observation Food Additives and Contaminants 18:644-646

Govaris A, Roussi V, Koidis P, Botsoglou N (2002) Distribution and stability of aflatoxin M1 during production and storage of yoghurt Food Additives and Contaminants 19:1043-1050

Guan D, Li P, Zhang Q, Zhang W, Zhang D, Jiang J (2011) An ultra-sensitive monoclonal antibody-based competitive enzyme immunoassay for aflatoxin M1 in milk and infant milk products Food Chemistry 125:1359-1364

Hussain I, Anwar J (2008a) A study on contamination of aflatoxin M1 in raw milk in the Punjab province of Pakistan Food control 19:393-395

Ito Y, Peterson SW, Wicklow DT, Goto T (2001) Aspergillus pseudotamarii, a new aflatoxin producing species in Aspergillus section Flavi Mycol Res 105:233-239

Kamkar A (2005) A study on the occurrence of aflatoxin M1 in raw milk produced in Sarab city of Iran Food control 16:593-599

Kamkar A, Fallah A, Mozaffari Nejad A S (2014) The review of aflatoxin M1 contamination in milk and dairy products produced in Iran. Toxin Review 33: 160–168.

Megalla S E, Hafez A H (1982) Detoxification of aflatoxin B1 acidogenous yoghurt Mycopathologia 77: 89-91.

Mohamed O (1998) Stability of AFM1 and AFB1 in yoghurt manufactured by Lactobacills Acidophilus and Bifidobacterium bifidum. Al-Azhar J Agric Res 27:104-120

Motawee M (2013) Reduction of aflatoxin M1 content during manufacture and storage of Egyptian domaiti cheese International Journal of Veterinary Medicine: Research and Reports 2013:207299

Motawee MM, Abd El-Ghany MA, (2011) Effect of Some Lactic Acid Bacteria Strains on Aflatoxins Reduction in Some Dairy Foods. The 6thArab and 3rd International Annual Scientific Conference, Faculty of Specific Education Mansoura University - Egypt April 13-14

Motawee MM, Bauer J, McMahon DJ (2009) Survey of aflatoxin M1 in cow, goat, buffalo and camel milks in Ismailia-Egypt Bull Environ Contam Toxicol 83:766-769.

No E (2006) Setting maximum levels for certain contaminants in foodstuffs Official Journal of the European Union L 364

Omeiza G K, Mwanza M, Enem S I, Godwin E, Adeiza M A, Okoli C (2018) Reducing Efficiencies of the Commonly Used Heat Treatment Methods and Fermentation Processes on Aflatoxin M1 in Naturally Contaminated Fresh Cow Milk Open Journal of Veterinary Medicine 8: 134-145

Oxoid manual (1998) The Oxoid manual of culture media: Oxoid Limited, Basiugtoke, Hampshire, England.

Prandini A, Tansini G, Sigolo S, Filippi L, Laporta M, Piva G (2009) On the occurrence of aflatoxin M1 in milk and dairy products Food and chemical toxicology 47:984-991

Rahimi E, Karim G (2008) Determination of aflatoxin M1 in raw milk producing in dairy farmsin Chahar-Mahal-Bakhtiari province using ELISA Journal of Food Science and Technology 5: 51–59.

Ranadheera R, Baines S, Adams M (2010) Importance of food in probiotic efficacy Food research international 43:1-7

Rasic J L, Skrinjar M, Markov S (1991) Decrease of aflatoxin B1 in yoghurt and acidified milk Mycopathologia 113: 117-119.

Sarımehmetoglu B, Kuplulu O, Celik TH (2004) Detection of aflatoxin M1 in cheese samples by ELISA Food control 15:45-49

SAS S. (2008) STAT 9.2 User's Guide. Version 9.2. Cary, NC: SAS Institute. Inc.

Sessou P, Farougou S, Yèhouenou B, Agbangnan P, Azokpota P, Youssao I, Sohounhloue D (2013) Investigation on aflatoxin M1 content of traditional cheese wagashi produced in Benin

Shahin A (2007) Removal of aflatoxin B1 from contaminated liquid media by dairy lactic acid bacteria Int J Agri Biol 9:71-75

Shils ME (1994) National Dairy Council Award for Excellence in Medical and Dental Nutrition Education Lecture, 1994: nutrition education in medical schools—the prospect before us. Oxford University Press,

Tajkarimi M, Aliabadi-Sh F, Nejad AS, Poursoltani H, Motallebi A, Mahdavi H (2008a) Aflatoxin M1 contamination in winter and summer milk in 14 states in Iran Food Control 19:1033-1036

van Egmond HP, Dragacci S (2001) Liquid Chromatographic Method for Aflatoxin M 1 in Milk Mycotoxin Protocols:59-69

van Egmond HP, Schothorst RC, Jonker MA (2007) Regulations relating to mycotoxins in food Analytical and bioanalytical chemistry 389:147-157

Yuanling L, Mark E, Baker B, Todd Y, Bonner Denton M (1996) Quantitative Analysis of Aflatoxins by High-Performance Thin-Layer Chromatography Utilizing a Scientifically Operated Charge-Coupled Device Detector. Analul Chemistry 68: 22 3885-3891

دور بكتيريا البروبيوتيك في الحماية ضد التلوث بالسم الفطري الأفلاتوكسين M1 في الحليب والمنتجات اللبنية.

محمد عبدالحميد ربيع[1]، السيد محمد عبدالواحد خليل[1]، جمال مصطفى محمد[2]، خالد مغاوري الزهار[3] و أحمد عبدالظاهر[4]

[1] قسم علوم الأغذية- كلية الزراعة- جامعة الزقازيق

[2] قسم الميكروبيولوجيا الزراعية- كلية الزراعة- جامعة الزقازيق

[3] كلية الزراعة والطب البيطري- جامعة القصيم- السعودية

[4] قسم الكيمياء- كلية العلوم- جامعة الزقازيق

أجريت الدراسة الحالية لدراسة التلوث بالأفلاتوكسين م1 (AFM1) في الحليب والجبن القريش واليوغورت. تم الكشف عن AFM1 في الحليب الخام في 10 عينات من 12 عينة (83.3 ٪) من كل موسم. كانت تركيزات AFM1 أعلى في فصل الشتاء من فصل الصيف، مع القيم الوسطية 311.8 و 207.0 نانوغرام/لتر خلال فصلي الشتاء والصيف، على التوالي. أوضحت النتائج أن 83.33٪ من عينات الجبن التي تم فحصها تجاوزت حدود الاتحاد الأوروبي (50 نانوغرام / كيلوغرام) ، وأن 70.83٪ من هذه العينات تجاوزت حدود الدستور الغذائي (500 نانوغرام / كجم) خلال فصلي الصيف والشتاء. من بين ثلاثة أنواع من الألبان التي تم اختبارها ، كان للجبن أعلى تركيز منAFM1 ، وكان للتركيز الزبادي أقل تركيز. خفضت المعالجة الحرارية تركيزات AFM1 في جميع عينات اللبن الخام التي تم اختبارها. عمل الغليان على الخفض منAFM1 بنسبة 26.71 ٪ ، في حين أن البسترة فقط خفضت بنسبة 15.45 ٪. في الزبادي، السلالتين من بكتيريا البروبيوتيك (Lactobacillus acidophilus and Bifidobacterium lactis) منفردة خفضت تدريجياً تركيز AFM1 كدالة للوقت في الحليب الملوث بـ 25 نانوجرام/كجم، مع التخلص التام بنهاية فترة التخزين المبردة (3 أيام)، بينما كان مزيج من سلالتين أفضل قدرة على تقليل مستوى AFM1 . كان الجمع بين كل من بكتيريا بروبيوتيك (*Lactobacillus acidophilus* and *Bifidobacterium lactis*) أكثر قدرة على تقليل AFM1في اللبن الملوث بـ50.0 نانوجرام/كجم أو 75 نانوجرام/كجم من AFM1. كانت التخفيضات الأكثر شمولاً لتركيز الـ AFM1 هي 41.80 نانوجرام/كجم (45.3٪) و 22.6 نانوجرام/كجم (69.90٪) و 7.12 نانوجرام/كجم (92.8٪)، والتي تم تحقيقها باستخدام نفس التركيز لكل سلالة على حدة وعلى التوالي، بعد يومين في الحليب الملوث بـ 75 نانوجرام/كجم. لم يتم ملاحظة وجود AFM1 بعد ثلاثة أيام باستخدام السلالات مجتمعة.