PREVALENCE AND DETECTION OF AFLATOXIN M1 IN DAIRY COW RAW MILK RAISED UNDER DIFFERENT BREEDING SYSTEMS, NUTRITION AND SEASON IN BEKAA VALLEY- LEBANON

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ABSTRACT

Aflatoxin M1 (AFM1) contamination in dairy products is a problem threatening public health in all areas of the world. The aim of this study was to identify the level of AFM1 in cow raw milk in three dairy farm regions in Bekaa Valley, Lebanon. A total of 352 raw milk samples were collected from October 2021 to August 2022. AFM1 was analyzed by ELISA. Only 25.2%, 55.8%, and 25% were reported free in Baalbeck, Zahleh and in West Bekaa respectively. The yearly mean AFM1 contamination in samples varies between 14.31 ng/kg, 11.5 ng/kg, and 10,5ng/kg in Baalbeck, West Bekaa, and in Zahleh respectively. The highest level was also recorded in small farms compared to large scale farms. A significant difference was seen in winter between seasons (p value 0.032) and in West Bekaa between regions (p value 0.012). Legislation and monitoring procedures must be implemented to reduce the exposure to this toxin.

Key words: Aflatoxin; AFM1; Dairy farm; ELISA; Raw milk.

Introduction

Aflatoxins are fungal toxins produced by certain species of *Aspergillus* especially *parasiticus*, but rarely by *A. nominus* (Rahimi *et al.*, 2010) which may grow on several kinds of agricultural products. The major type of naturally occurring AFs have been identified: Aflatoxin B1 (AFB1), Aflatoxin B2 (AFB2), Aflatoxin G1 (AFG1), Aflatoxin G2 (AFG2), Aflatoxin M1 (AFM1) and Aflatoxin M2 (AFM2). AFB1 represents the highest degree of toxicity followed by AFM1, AFG1, AFB2 and AFG2 (Gourama and Bullerman, 1995). AFB1 is considered by the International agency for research on cancer to be the most hepatocarcinogen, teratogen and mutagen of this group of mycotoxins (Gourama and Bullerman, 1995), (IARC, 2002). AFM1, the hydroxylated metabolites of AFB1, may be found in milk, milk products and meat of dairy cattle and mammals that have ingested the feedstuffs contaminated with AFB1 (Creppy, 2002). Milk has the greatest demonstrated potential for introducing AFM1 into the human diet and exposure to AFM1 through milk products is a serious problem for public health (Ruangwises and Ruangwises, 2010). Several surveys on AFM1 contamination and its prevalence in milk and dairy products have recently been done in Lebanon (ElKhoury *et al.*, 2011), (Elkak *et al.*, 2011), Hassan and Kassaify (2014), and (Daou *et al.*, 2020). Similar study have been done in Syria (Ghanem and Orfi, 2009), in Palestine (Al-Zuheir and Abo Omar, 2012), in Jordan (Natour *et al.*, 1991) and Sharaf, (2012), in Kuwait (Dashti *et al.*, 2009), in Turkey (Celik *et al.*, 2005). Due to the potential toxicity of AFM1, most countries have set maximum permissible levels for AFM1 in milk and milk products. Maximum permissible levels of aflatoxins M1 in milk are 0.05 ng/g in the European Union and 0.5ng/g in the United States. Other study have been done to show the influence of different breeding systems, feeding, and season effect on the variations in AFM1 levels in milk (Lopez *et al.*, 2003). There is variation with the level of AFM1 from animal to animal, from day to day and from one milking to the next (Martins and Martins, 2004). Milk yield is one of the factors affecting the total excretion of AFM1 (Masoero *et al.*, 2007). Previous research has found a significant seasonal change in AFM1 levels in milk (Kamkar, 2005; Rahimi and Ameri, 2012; Ruangwises and Ruangwises, 2010). It has been found that the levels of AFs in feed are greater during wet seasons than during dry seasons. Furthermore, the usage of large volumes of contaminated concentrates is more common during the winter months (Kamkar *et al.,* 2011).

The present study aims at detecting the level of AFM1 in bovine raw milk in different districts of Lebanon Bekaa valley during a one year under different management systems and in the different seasons of the year.

Materials and methods

Experimental location, animals and farms

The study have been done in the region of Bekaa. The Bekaa Valley known in classical antiquity as Coele-Syria, is a fertile Valley and main agricultural area in eastern Lebanon. The three main dairy regions with the different farms that are included in our study of the Bekaa valley (R1: **Baalbeck**, R2: **Zahleh**, and R3 **West Bekaa**) are characterized by different farming types and by different microclimates (Wikipedia, 2023).

Milk samples collection

In each region R1, R2, and R3 of the study: 30, 29, 29 farms have been selected respectively and divided as small scale farms: less than 20 cows/farm, and large scale farms: more than 20 cows/farm. **A total of 352 milk samples**: 116, 120, 116 samples were collected from the farms of the three regions (R1), (R2) and (R3) respectively. Milk samples have been collected from the tank of each farm on seasonally basis (Mid-season) from October 2021 till August 2022. The samples were transported to the laboratory in an insulated container at about 4°C. The samples were kept at -20°C deep-freeze till tested. At the time of analysis samples were brought up to room temperature (Richard *et al.*, 1993). An inspection of the feed storage sides was carried to determine the physical condition of the stores plus the presence of extrinsic factors that can influence the quality of feeds. All samples were subject to ELISA test at the laboratories of Dairy Khoury.

ELISA test procedure, and statistical analysis

The quantity of AFM1 was determined according to Enzyme-linked Immuno Sorbent Assay (ELISA) by using the RIDASCREEN® Aflatoxin M1 (R1121) (R-biopharm, Darmstadt, Germany) test kit which is a competitive enzyme immunoassay based on antigen-antibody reaction. Special software, the RIDA®SOFT win (Art. No. Z9999) is available to evaluate the RIDASCREEN enzyme immunoassay. Statistical analysis was conducted using Statistical Package for the Social Science (SPSS version 8.0 for Microsoft Windows; SPSS, Chicago, IL). Results were expressed as mean ± standard deviation. Analysis of variance ANOVA was conducted to determine differences in AFM1 content among milk sources, systems, and seasons. A probability of <0.05 was considered significant.

Results

The level of AFM1 in raw cow milk in Baalbeck during different seasons in different farms showed a minimum value in fall in both small and large scales farms and maximum values in winter in the 2 systems. In the small-scale farms and large scale farms, no samples showed AFM1 higher than the European Standard (50 ng/kg) while all the milk samples in fall were lower than the European Standard.

The results in Zahleh region in small scale farms revealed that AFM1 was found in 11.1% of the milk samples in spring, were found higher than the European Standards in spring (50ng/Kg). In large scale farms, 5% of the tested samples in spring showed AFM1 values higher than the European standard. None of the samples tested in fall, summer or winter showed AFM1 level higher than the European standards.

Concerning AFM1 content in raw milk in West Bekaa regions in the four seasons there is no significant difference (P>0.05) in mean AFM1 values between the 2 systems and the four seasons in this region. In small scale farm, the incidence of AFM1contamination ranges from 0 in spring to 15.3% of milk samples in summer. In large scale farms, only 2 of the tested samples were higher than the European standards in summer while none of the tested samples in winter and fall and spring showed AFM1 values higher than the European standards.

As overall the level of aflatoxin M1 contamination recorded in this study was as (table 1) follow: 1.73 % of the analyzed samples in small scale farms show AFM1 level higher than the European standards (ES). 1.63 % of the analyzed milk samples in large scale farms show a level of contamination higher the European Limits of 50 ng/kg. The mean AFM1 level in analyzed samples shows the lowest level in fall (2.96±5.4 ng/kg and 2.52±4.68 ng/kg) in the 2 systems small and large scale respectively and the highest values in summer (19.3±15 ng/kg vs 15.29±12.9 ng/kg) for the 2 systems respectively.

Table 1: AMF1 level during different seasons and farm scale (n: number; ES: European standard)

		Samples Size (n)	Samples (n) > 50 ng/kg (ES)	% of Samples > 50 ng/kg (ES)	Mean±SD (ng/kg)
Winter	Small scale	43	0	0	14.71±8.9
	large scale	45	0	0	14.7±8.9
Spring	Small scale	43	1	2.33	13.3±10.8
	large scale	45	1	2.14	16.24±15.08
Summer	Small scale	43	2	4.6	19.3±15
	large scale	45	2	4.37	15.29±12.9
Fall	Small scale	43	0	0	2.96±5.4
	large scale	45	0	0	2.52±4.68
Overall	Small scale	172	3	1.73	12.25±10.2
	large scale	180	3	1.63	12.05±12.4

Effect of season on AFM1 level in raw bulk milk samples in Bekaa

88 samples were analyzed with competitive ELISA in each season. Of the 88 samples analyzed, 80% of samples were found to be contaminated with AFM1 in winter season (AFM1 above 50 ng/kg), 22% of samples in fall, 78.7% of samples in spring and 83% of samples in summer. On the other hand, 5% failed to the desired level of the European communities and Codex (50 ng/kg) in winter. The number of AFM1 positive milk samples above European standards were 2.5% of samples in spring and 3.65% of samples in summer.

Effect of regions and raising systems on Milk AFM1 contamination

The mean concentration of milk AFM1 showed a significant difference (p<0.05) in between the different districts of Bekaa valley with the highest values recorded in West Bekaa and the lowest AFM1 values recorded in milk samples from Baalbeck. The three districts of the study showed a higher AFM1 contamination level in winter (table 2).

Table 2: Mean concentration of AFM1 (ng/kg) in milk in the three different regions and seasons

Region	Winter	Spring	Summer	Fall
Baalbeck	18.7±7.78[a]	16.8±7.6	20.9±12.8	1.56±4.5
Zahleh	14.13±12.14[a]	13.24±19.16	13.55±10.8	2.78±5.21
West Bekaa	11.63±9.35[b]	14.37±10.06	17.17±17.36	3.88±5.23

The percentages distribution of milk samples according to their levels of AFM1 contamination are presented in table 3. Out of the all samples analyzed in each district, only 25.2% were reported free in Baalbeck, 55.8% in Zahleh and 25% in West Bekaa. The percentages of milk samples that fail to meet European standards were as follows: 0% in Baalbeck, 1.5% in Zahleh and 3.1% in West Bekaa (figure 1).

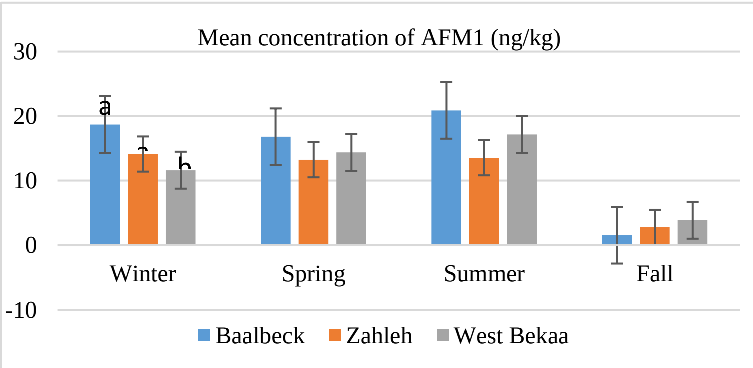

Figure 1: Mean concentration in (ng/kg) of AFM1 (Y axis) in different regions and seasons (X axis)

Table 3: % of AFM1 contamination in milk samples (ND: not detectable, ES: European standard)

	Free samples	**% of Contaminated Samples**				
		Below ES (< 50 ng/kg)			**Above ES (> 50 ng/kg)**	
Regions	ND <5 ng/kg	5-10 ng/kg	10-25 ng/kg	25-50 ng/kg	50-80 ng/kg	> 80 ng/kg
Baalbeck	25.2 %	12.5%	42.2%	20.1%	0%	0%
Zahleh	55.8%	16.4%	17.1%	9.3%	1.5%	0%
West bekaa	25%	25.7%	35.9%	11.8%	2.34%	0.8%

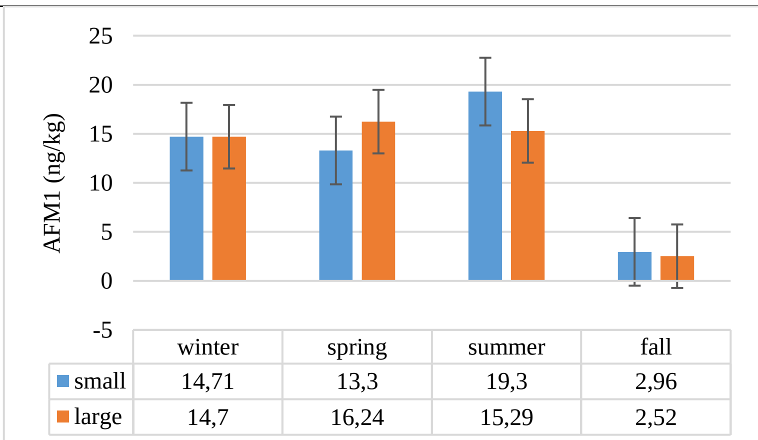

Figure 2: Incidence of AMF1 during different seasons and farm scale

According to figure 2 and table 1, the incidence of AFM1 contamination in milk in this study was higher in small scale farms compared to large scale farms with higher values recorded in summer in the 2 systems.

Discussion

These results of the present study are slightly higher than those reported by Hassan and Kassaify (2014) who reported a mean AFM1 content of 10.74±2.01ng/L in cow raw milk collected from different regions of Lebanese markets. In another study in Lebanese cow raw milk, Elkhoury *et al.* (2011) reported a contamination level of 40.62% (26/64 above 5 ng/L) with a range of AFM1 level between 0.005µg/L to 0.05µg/L. In a preliminary study conducted by Elkak *et al.* (2011) to determine the occurrence of AFM1 in 77 cow and goat milk samples (38 raw milk, 25 pasteurized milk and 14 powder milk samples); obtained either from local small farms, or markets. The revealed rates of AFM1 contamination were 73.6%, 68.0%, 35.7% for the raw, pasteurized and powder milk samples, respectively. The individual values, within each category of milk samples, ranged from 2.63 to 126 ng/l (mean ±SD = 60 ng/l), 3.27–84.4 ng/l (mean ±SD = 30.6 ng/l) and 9.18–16.5 ng/l (mean ±SD = 13.7 ng/l) for the raw, pasteurized and powder milk samples, respectively. Of the positive samples, 29 were still below the permitted limit (50 ng/l) set by the European Commission whereas 21 exceeded the permissible limit. In other recently study conducted by Daou *et al.* (2020), results showed contamination in raw milk, pasteurized and UHT milk, and dairy products at a range of 0.011–0.440 µg/L, 0.013–0.219 µg/L, and 0.015–7.350 µg/L respectively; with 28%, 54.5%, and 30 45.5% respectively of samples with AFM1 above maximum tolerable limit (MTL) set by the European Commission. Regarding results of the above studies, these findings shows that AFM1 contamination levels in milk differ among regions and farms especially with high level of AFM1 in small farms. Similar studies conducted in neighboring countries showed also high contamination level. In Syria, Ghanem and Orfi (2009) studied the incidence of contamination of AFM1 in milk samples collected from the Syrian. A total of 126 samples composed of raw cow milk (74 samples), raw sheep milk (23), raw goat milk (11), pasteurized cow milk (10) and powdered milk (8) showed that 80% of tested samples were contaminated with various levels of AFM1 ranging from >20 to 765 ng/l. Percentages of AFM1-contaminated samples exceeding the American (500ng/kg) and European tolerance limits (50ng/kg) were 22% and 52%, respectively. In Palestine, AL- Zuheir and Abo Omar (2012) conducted a study to highlight the occurrence of AFM1 in Palestine raw milk collected at farms from Tulkarm, Nablus and Jenin.. There was a high incidence rate with 92 % (11 of 12) and the highest means of contaminated with AFM1 in the samples tested in Tulkarm city ($P \leq 0.05$). 20 % of the analyzed samples (8 of 40) exceeded the maximum permissible limit (50 ppt) in European Codex. In Jordan, Sharaf (2012) undertake study to determine the presence of aflatoxin M1 (AFM1) in animal milk and the. A total of 100 samples of fresh animal milk (cows, goats, camels and sheep) and fermented milk (buttermilk) were collected during 2010-2011 years. The concentrations of AFM1 in 70 samples from fresh and fermented milk were higher than the maximum tolerance limit accepted by European Union and USA (50 ng/kg). However, a lower milk AFM1 contamination level has been reported in Jordan in 1991 by Natour *et al.* (1991). In Kuwait, 54 samples of dairy products were analyzed for aflatoxin M1, 28% were contaminated with AFM1 (Dashti *et al.*, 2009). In Turkey, in Celik *et al.* (2005) study Seventy-five samples (88.23%) were found to be contaminated with AFM1, and 48 samples (64%) exceeded the legal level of AFM1 in milk according to the Turkish Food Codex and Codex Alimentarius limit (50 ng/kg). Rastogi *et al.* (2004) reported that 75% of liquid milk samples exceeded European Communities and Codex Regulations.

The trace occurrence of aflatoxin is a critical topic, because of the vital daily consumption of milk in an agricultural community like in Bekaa valley, especially by infants and children. Since aflatoxin M1 is a metabolite of aflatoxin B1 excreted in milk, detecting high concentrations of aflatoxin M1 in raw milk samples implies the presence of very high aflatoxin B1 levels in feed, particularly in hay. Many factors may affect the level of aflatoxin B1 in animal feeds. Geographic and climate changes can affect the farm management practices and feed quality. These effects can lead to the wide variations in aflatoxin M1 levels in milk (Zaki *et al.*, 2012).

Moreover, the current study found that AFM1 levels in milk samples from small farms were greater than those from large farms. This was consistent with field observations and might be explained by the concept that excellent storage techniques and cleanliness requirements are not strictly followed on traditional farms. Furthermore, farmers are unaware of the possibility of mycotoxins contaminating animal feed.

This study showed also a significant difference in the level of milk AFM1 contamination between the different districts of Bekaa valley with the highest AFM1 contamination recorded in West Bekaa while the lowest one was registered in Baalbeck. This difference could be attributed to difference in climatic condition or to difference in feeding practices. The high milk AFM1 contamination level in West Bekaa may be due to the wet winter seasons, the dairy cattle farmers in Bekaa valley harvest hay in the summer, store it until the next season, and feed it to the cattle during the year. This enhances the growth and aflatoxin B1 production from fungi present in haystacks, stimulated by the high humidity, high temperature, and inappropriate storage conditions. Martins and Martins (2004) reported that about 1–2% of AFB1 in animal feed is transformed to AFM1 in milk with variations from animal to animal, from day to day and from one milking to the next. When the intake of AFB1 is stopped, the AFM1 concentration in the milk decreases to an undetectable level after 72 hours. Moreover, Galvano *et al.*, (2005) reported that 0.3-6% of ingested AFB1 is available as AFM1 in milk. Many studies in the world reported the occurrence of AFM1 in dairy products and evidence of potential hazardous human exposure, as milk is a key source of nutrients for humans (Galvano *et al.*, 1996, Heshmati and Milani, 2010). The lower level of aflatoxin M1 contamination in Zahleh could be attributed to better climatic condition than west Bekaa and Baalbeck and the better knowledge in terms of storage techniques, more advanced farms and feed mills, mandatory addition of mycotoxin binders too. This climatic condition is not optimal to fungal growth. The low AFM1 level in this area could be attributed to the low milk production of local breed raised in this region. Masoero *et al.* (2007) suggested that milk yield is one of the factors affecting the total excretion of AFM1. High yielding dairy cows with a production up to 40 liters of milk per day showed a carry-over percentage as high as 6.2%.

The low level of AFM1 in Zahleh could be related to the opportunity of cows to have access to green forages and grazing outside in summer and spring seasons. In fact, the most common ingredients of rations fed to dairy cows as the farmers informed us were corn silage, wheat , barley straws and hay, and the concentrates dominantly contained maize, barely, wheat bran, soybean meal, cottonseed meal and full TMR rations for many large scale farms.

Since the AFM1 appears in milk, followed by ingestion of AFB1-contaminated feed, feedstuff quality is an essential factor in production of contaminated milk. Therefore, the wide fluctuations in AFM1 concentrations in this could be associated with dairy cattle feed quality. On the other hand, the feed quality is affected by many factors such as geographic and climatic conditions, feeding system types and farm management practices (Lopez *et al.*, 2003). Any changes in these factors could lead to marked fluctuations in AFM1 levels in milk.

The Milk AFM1 contamination showed a seasonal fluctuation with highest contamination level in summer (83% of the samples with 3.65% above European standards) and lowest level in fall (22% of the samples with 0% above the European standards). This result could be related to prolonged good weather and temperatures higher than yearly mean ±SDs during the month of October 2021, and to the deteriorated quality of stocked grains for about one year in Ukraine that reached Lebanon in summer 2022 as well as the reduction of economical ability of farmers to add high quality products to the feed such as mycotoxin binders, premixes and to maintain the storage areas intact.

A marked seasonal variation in AFM1 levels in milk has been previously reported (Kamkar, 2005; Rahimi and Ameri, 2012; Ruangwises and Ruangwises, 2010). It has been reported that AFs levels in feed are higher in rainy than in dry seasons. Moreover, the use of high amounts of contaminated concentrates is more frequent in cold months (kamkar *et al.*, 2011). The results of the present study show that the mean concentrations in raw milk samples collected in autumn were higher than in other seasons. Such variation may be a result of toxin accumulation when storage occurs in hot and humid conditions. Many authors (Blanco *et al.*, 1988; Lopez *et al.*, 2003; Kamkar, 2005) reported on a higher number of yeasts, moulds and consequently on a higher concentration of mycotoxins in ensiled feed, mostly used in autumn or winter. Tajkarimi *et al.*, 2008 reported also higher level of AFM1 in cold seasons compared to hot seasons. The reason is that in winters milking animals are usually fed with compound feeds and thus concentration of AFB1 increases, which in turn increases AFM1 concentration in milk. In addition, humidity affects the presence of AFB1 in feeds. *A. flavus* and *A. parasiticus* can easily grow in feedstuffs having humidity between 13% and 18%, and then they are able to produce aflatoxin in environmental humidity between 50% and 60% (Jay, 1992). For this reason, the level of AFB1 in feed in rainy months is more than dry months, which is in agreement with our study results in September-November compared with the other months. This might be due to hot summer in Bekaa and raining in September that increase aflatoxin production by the end of summer.

Conclusion and recommendation

High levels of AFM1 in milk and other dairy products are considered undesirable because it has toxic, teratogenic and carcinogenic properties. Considering the results, this survey revealed high level of aflatoxin M1 contamination in cow milk in different districts of Bekaa Valley. The highest level of aflatoxin M1 in raw cow milk was recorded in West Bekaa and the lowest level in Baalbeck. Higher level of aflatoxin M1 was also recorded in small holder livestock farms compared to large scale farms. Finally, higher level of aflatoxin M1 contamination in cow milk was recorded in winter, spring and summer compared to fall. Additional research must be conducted on a regular basis to assure the safety of local dairy products. Legislation and monitoring procedures from government agencies must be implemented to reduce the exposure of the Lebanese people to this toxin.

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