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

ABSTRACT OF A DISSERTATION

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The dissertation is written on 156 pages and contains 20 tables and 24 figures and 3 appendix. The list of references includes 382 titles. A total of 16 conclusion and 6 recommendations were made and 5 scientific and applied contributions.

The dissertation defense will be held on January 10 21, 2025, at 11.00 in the Academic Hall "M. Dakov" at Building A of the University of Forestry, Sofia, 10 Kliment Ohridski Blvd. at an open meeting of a scientific jury approved by Order No. 646/8.11.2024 of the Rector of the University of Forestry with the following members:

Chairman: Prof. PhD Krasimira Ivanova Genova

Members: Assoc. Prof. PhD Metodi Hristov Petrichev
Prof. PhD Rumen Georgiev Binev
Assoc. Prof. PhD Anton Georgiev Rusenov
Assoc. Prof. PhD Ivan Balchev Trifonov

The materials on the defense (dissertation, abstract, reviews and opinions) are available to those interested on the website of the University of Forestry (www.ltu.bg) and in the Dean's office of FVM – Sofia, 10 'Sveti Kliment Ohridski' Blvd.

I. INTRODUCTION

Aflatoxins are fungal toxins produced by certain species of Aspergillus especially *A. 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). According to an animal model, the conversion factor of AFB1 into AFM1 ranges from 0.3 to 6% (Var and Kabak, 2009). According to the Food and Agriculture Organization (2004) the maximum allowable amount of AFB1 in feed was established at 20 ppb. United State of America (USA) Food and Drug Administration (FDA) imposed an aflatoxin limit of 20 ppb for foods, as well as for the majority of feeds and feed ingredients. On the other hand, the European Union (EU) set a 20 μg/kg maximum limit for AFB1 in all feed materials, complete and complementary feeding stuff for cattle, sheep, goats, pigs, and poultry (except for young animals), and 5 μg/kg for complete feeding stuff for dairy animals (Food Standard Agency (FSA), 2004)).

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 0.5 ng/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).

II. PURPOSE AND TASKS

Purpose

The purpose of this study was to identify the level of aflatoxin M1 (AFM1) in cow raw milk produced in three different dairy regions in Bekaa Valley, Lebanon (Baalbek, Zahleh and West Bekaa) characterized by different farming types and by different microclimates over one year.

Tasks

To achieve the purpose above the following tasks were set:

1. To categorize and select appropriate cow farms in the three dairy regions of the Bekaa Valley (Baalbek, Zahleh and West Bekaa).
2. To evaluate the influence of regional differences on the aflatoxin M1 (AFM1) raw cow milk contamination.
3. To evaluate the influence of the farming type and technology on the aflatoxin M1 (AFM1) raw cow milk contamination.
4. To evaluate the seasonal fluctuations in the aflatoxin M1 (AFM1) raw cow milk contamination.
5. To evaluate the occurrence and levels of aflatoxin B1 (AFB1) in most commonly used feedstuffs as source of aflatoxin M1 (AFM1) raw cow milk contamination.

III. MATERIALS AND METHODS

III.1. DESCRIPTION OF THE EXPERIMENTAL SITE

The study have been done in the region of Bekaa. The Bekaa Valley and known in classical antiquity as Coele-Syria, is a fertile Valley and main agricultural area in eastern Lebanon. It is Lebanon's most important farming region. Industry also flourishes in Bekaa, especially that related to agriculture (Fig. 2).

The Bekaa is located about 30 km east of the Capital Beirut. The Valley lies between two parallel ranges of mountains, Mount Lebanon to west and the Anti-Lebanon Mountains to the east (Fig. 1). It is the northern continuation of the Jordan Rift Valley, and thus part of the Great Rift Valley which stretches from Syria to the Red Sea. It is only about 120 Km in length and has an average width of about 16 Km.

The three main dairy regions with the different farms that are included in our study of the Bekaa valley (R1: **Baalbeck** (Fig. 3), R2: **Zahleh** (Fig. 4) and R3 **West Bekaa** (Fig. 5)) are characterized by different farming types and by different microclimates. R1 is located at an altitude of 1250 m and has a semi-arid climate (410 mm precipitation/year), R2 region (900 m of altitude and average rainfall 686 mm/year) and R3 (average rainfalls of 860 mm/year) are considered as temperate climate (Wikipedia, 2023).

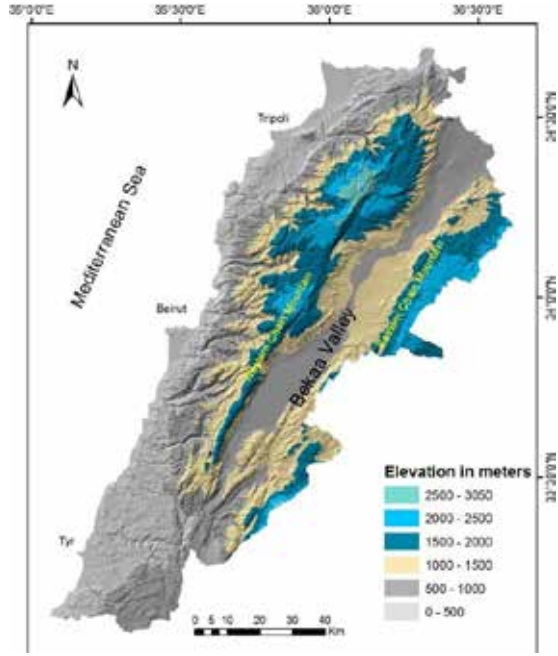

Fig. 1. Lebanon map showing the localisation of the Bekaa Valley between the two mountains (Google maps, 2022).

Fig. 2. A view across the Bekaa Valley (photo by the author, 2022)

Fig. 3. Geographical location of Baalbeck area (R1: Red line) (Google Maps, 2022).

Fig. 4. Geographical location of Zahleh (R2: Red line) (Google Maps, 2022).

Fig. 5. Geographical location of West Bekaa (R3: Red line) (Google Maps, 2022).

III.2. EXPERIMENTAL ANIMALS AND FARMS

The number of milking cows included in the study of each region (R1, R2, and R3) is mentioned in Table 1.

Table 1. Total milking cows in each region in relation with farm scaling and total milk samples

	R1: Baalbeck	R2: Zahleh	R3: West Bekaa	Total
Total milking cows included in the study	2417	1655	1285	
Small scale farm	21	9	13	
Large scale farm	9	20	16	
Milk Samples = Total farm (for 1 season)	30	29	29	
Total Milk Samples for All Seasons	120	116	116	352

The majority of herd raised in all regions belong to the local (Baladi) breed characterized by low milk production; Holstein, Holstein x Baladi and Normandy breeds are noticed too especially in Zahleh and Baalbeck (Fig. 6).

Fig. 6. Cow breed of the experiment (photo by author, 2021).

III.3. FEEDING PRACTICE

In the three dairy regions, concentrates composed of corn grains, barley bran, soybean, wheat bran, and cotton seeds represented the main portion of the diet. In fall and winter and in R1, R2 and R3 cows received wheat straw in addition to concentrates, whereas the herd of R2 and R3 received corn silage (Fig. 7). However, in spring and summer, cows in R3 region received less concentrates and grazed more on the available pasture while in R1 and R2 regions, cattle received alfalfa and hay in spring and summer season. Moreover, most cows in the three different dairy regions calved in spring and the highest production was seen during this period.

Fig. 7. Feed storage in different farms (photo by author, 2021).

III.4. MILK SAMPLES AND FEEDS INGREDIENTS COLLECTION

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 (table 1 and Annexe 1, 2, 3). Some Farms have been removed from the study in each region (B9 and B10 from R1; Z4, Z31, Z32 from R2; and WB13, WB14, WB 21 from R3) because the owners

could no longer buy fodder, due to the economic crisis and situation in Lebanon during the period of the study till present, so they had to sell their farms (Annexe 1, 2, 3).

Fig. 8. Milk samples collection (photo by author, 2021)

A total of 352 milk samples: 116, 120, 116 samples were collected from the farms of the three regions Baalbeck (R1), Zahleh (R2) and West Bekaa (R3) respectively (Table 12). 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 (Fig. 8). 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-Ain Sendyeneh-Mont Liban.

Feedstuffs samples collection: 15 farms from three regions (5 farms from Baalbeck (R1), 5 from Zahleh (R2), and 5 from West Bekaa (R3)) was selected, and from each farm 6 feeds ingredients was collected (**To-**

tal: 90 samples). Those ingredients are Barley bran, Soya bean, corn, cotton seeds (imported), wheat bran and hay (local). For all ingredients, the sampling is from the farm not from the origin source. All samples were collected between the end of June 2022 and the beginning of July 2022. Most of the farm that were collected the sample are small scale farm from the three region. Samples were placed in plastic bags (200 g of each ingredient) and transported to the laboratory within 12 h. The samples were stored at 4°C and analysed for AFB1 within seven days after collection.

III.5. ELISA TEST PROCEDURE FOR DETERMINATION OF AFM1 AND AFB1

The quantity of aflatoxin M1 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. Such kit includes microtiter plates coated with capture antibodies, AFM1 standard solutions used for the construction of the calibration curve, peroxidase-conjugated AFM1, substrate (urea peroxidase), chromogen (tetramethyl-benzidine) and stop reagents 1 N sulfuric acid. A sample unit of 100 µL was used for the quantitative analysis of AFM1 using the commercial kit RIDASCREEN® (R-Biopharm, Germany). The milk samples were centrifuged for 10 minutes for 3500 rpm in 10°C. The upper creamy layer was completely removed by aspirating through a pastor pipette.

III.5.1. Test procedure

Accurate washing is very important. Do not allow microwells to dry up totally and avoid prolonged intervals between the working steps. Reproducibility in any ELISA is largely dependent upon the consistency with which the microwells are washed. Carefully follow the recommended washing sequence as outlined in the ELISA test procedure. Avoid direct sunlight during all incubations. Therefore, covering the microtiter plates is recommended.

1. Insert a sufficient number of wells into the microwell holder for all standards and samples to be run. Record standard and sample positions.
2. Pipet 50 µl of standard or prepared sample into separate wells; use a new pipette tip for each standard or sample.

3. Add 50 µl of enzyme conjugate (red cap) to each well.
4. Add 50 µl of anti-aflatoxin M1 antibody solution (black cap) to each well. Mix gently by shaking the plate manually and incubate for 10 min (+/- 1) at room temperature (20-25°C/68-77°F).
5. Dump the liquid out of the wells into a sink. Tap the microwell holder upside down onto a clean filter towel (3 times in a row) to remove all remaining liquid from the wells. Using a wash bottle or multichannel pipette, fill the wells (250 µl per well) with washing buffer. Empty the wells again and remove all remaining liquid. Repeat the washing step 2 more times.
6. Add 100µl of substrate/chromogen (brown crap) to each well. Mix gently by shaking the plate manually and incubate for 5 min (+/-0, 5) at room temperature (20-25°C/68-77°F) in the dark.
7. Add 100µl of stop solution (yellow cap) to each well. Mix gently by shaking the plate manually and measure the absorbance at 450 nm. Read within 10 min.

Concerning Sample preparation and test implementation of the feeds ingredients for AFB1 detection: The sample preparation (extraction, filtration, dilution) was done according to RIDASCREEN®FAST Aflatoxin SC Kit (Art. No.: R9002) wich is a competitive enzyme-linked immunoabsorbent assay for the quantitative analysis of aflatoxin in feed. The samples should be stored in a cool place, protected from light. A representative sample (according to accepted sampling techniques) should be ground and thoroughly mixed prior to proceeding with the extraction procedure. Bring all reagents and samples to room temperature (20 – 25°C) before use and perform sample preparation at room temperature. 5 g of ground sample was added to 25 ml of 70% methanol and shacked vigorously for three minutes with an automatic shaker. The mixture was filtered through Whatman No. 1 filter. 1 ml of the obtained filtrate was diluted with 1 ml of distilled or deionized water. 50 µl of the diluted filtrate per well in the test was used. Test preparation and test procedure are done according to the instructions and recommendations of the manufacturer of RIDASCREEN®FAST Aflatoxin SC Kit (R-Biopharm, Darmstdat, Germany).

III.5.2. Results of ELISA test for AFM1 and AFB1

For AFM1, special software, the RIDA®SOFT win (Art. No. Z9999) is available to evaluate the RIDASCREEN enzyme immunoassay. For single determination we recommend logit log evaluation and for double or multiple determinations cubic spline should to be used.The course of the standard curve is shown in the quality assurance certificate enclosed in the test kid. For AFB1, Special software the RIDASOFT® Win.NET (Art. No. Z9996FF) was used to evaluate the Ridascreen enzyme immunoassay. From this the software program calculates the standard Curve and the content of aflatoxin in the samples.

III.5.3. Remarque for the calculation without software for AFM1

Absorbance standard (or sample)/absorbance zero standard x 100 = % absorbance. The zero standard is thus made equal to 100% and the absorbance value are coated in percentage. The value calculated for the standards are entered in a system of coordinate on semi logarithmic graph paper against the aflatoxin M1 concentration [ng/l] (Fig. 9). In order to obtained the aflatoxin M1 concentration in ng/l (ppt) actually contained in sample, the concentration read from the calibration curve most be further multiplied by the corresponding dilution factor (Fig. 10). When working in accordance with the regulations stated, the dilution factor is as below:

Milk…….1
Milk powder (referring to reconstituted milk) …….1
Milk powder (referring to g-weight) ……10

Fig. 9. Milk analysis after centrifugation and microwells for ELISA testing (photo by author 2021)

Figure 10 : ELISA procedure using microwells and micropipettes and ELISA reader to see results (photo by author 2021).

III.6. STATISTICAL ANALYSIS

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.

For AFB1, All statistical analyses were performed in GraphPad Prism 8.4.2. We applied One-sample t-test to compare the mean concentration of AFB1 in feedstuffs to the USA and Lebanese legal limit (20 µg/Kg), and to determine if they are significantly different. Analysis of Variance (ANOVA) was employed to investigate for statistical differences among feedstuffs AFB1 mean levels. A probability of $p < 0.05$ was considered significant.

IV. RESULTS

IV.1. LEVEL OF AFLATOXIN M1 IN BULK RAW MILK IN DIFFERENT REGIONS OF BEKAA VALLEY

A. Level of aflatoxin M1 in raw cow milk in Baalbeck

The incidence of aflatoxin M1 contamination in milk in Baalbeck is shown in Table 2.

Table 2. Level of aflatoxin M1 in raw cow milk in Baalbeck during different seasons in different farms (n: number; ES: European standard)

					AFM1 : Concentration (ng/kg)		
Season	Farms	Samples Size (n)	Samples (n) > 50 ng/kg (ES)	% of Samples > 50 ng/kg (ES)	Minimum	Maximum	Mean±SD
Winter	small scale	21	0	0	6	31	22.11±8.6
	large scale	9	0	0	6	38	17.33±7.06
Spring	small scale	21	0	0	8	24	15.33±5.05
	large scale	9	0	0	10	44	20.33±10.71
Summer	small scale	21	0	0	0	47	21.95±13.47
	large scale	9	0	0	0	31	18.56±11.5
Fall	small scale	21	0	0	0	17	1.44±4.3
	large scale	9	0	0	0	16	1.76±4.6

According to table 2, the mean values of aflatoxin M1 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 Standards.

B. Level of aflatoxin M1 in raw bulk milk in Zahleh (Table 3)

Table 3: Level of aflatoxin M1 in Zahleh during different seasons in different farms (n: number; ES: European standard)

					AFM1 : Concentration (ng/kg)		
Season	Farms	Samples Size (n)	Samples (n) > 50 ng/kg (ES)	% of Samples > 50 ng/kg (ES)	Minimum	Maximum	Mean±SD
Winter	small scale	9	0	0	0	38	13.44±12.5
	large scale	20	0	0	0	20	15.15±12.13
Spring	small scale	9	1	11.5	0	63	10.33±20.9
	large scale	20	1	5	0	71	14.60±19
Summer	small scale	9	0	0	0	26	10.22±10.8
	large scale	20	0	0	0	38	15.05±10.7
Fall	small scale	9	0	0	0	10	1.1±3.33
	large scale	20	0	0	0	16	2.7±5.1

The results of this study in this 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 (50 ng/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.

C. Level of aflatoxin M1 in raw milk in West Bekaa

The aflatoxin M1 content in raw milk in West Bekaa regions in the four seasons is presented in table 4. However, there is no significant difference ($P > 0.05$) in mean AFM1 values between the 2 systems and the four seasons in this region.

Table 4. Level of aflatoxin M1 in raw cow milk in West Bekaa during different seasons in different farms (n: number; ES: European standard)

					AFM1 : Concentration (ng/kg)		
Season	Farms	Samples Size (n)	Samples (n) > 50 ng/kg (ES)	% of Samples > 50 ng/kg (ES)	Minimum	Maximum	Mean±SD
Winter	small scale	13	0	0	0	23	12.6±7.8
	large scale	16	0	0	0	38	11±10.8
Spring	small scale	13	0	0	0	26	12.3±7.3
	large scale	16	0	0	0	46	11±16.9
Summer	small scale	13	2	15.3	0	59	21.3±13.3
	large scale	16	2	15.3	0	80	18.7±11.5
Fall	small scale	13	0	0	0	13	5.38±6.1
	large scale	16	0	9	0	12	3.37±4.6

In small scale farm, the incidence of AFM1 contamination 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.

IV.2. EFFECT OF SEASON ON AFM1 LEVEL IN RAW BULK MILK SAMPLES IN BEKAA

The evolution of AFM1 contamination in milk samples in the three regions of the study throughout the year is illustrated in Fig. 11.

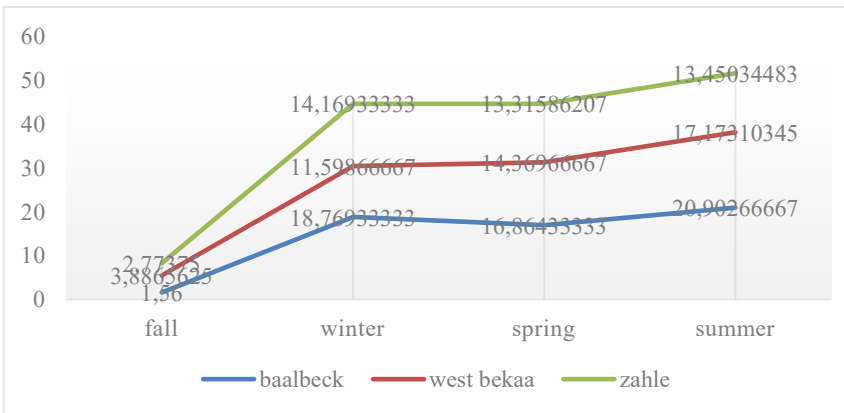

Fig. 11. Evolution of AFM1 levels during the year in the three regions

88 samples were analyzed with competitive ELISA in each season. The occurrence of AFM1 in raw milk samples in each season is shown in Table 5. 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.

Table 5. Percentage of AFM1 contaminated samples during different seasons (ND: not detected, ES: European standard)

	Free samples	AFM1 contaminated samples				
		Below European standards			Above ES	
	ND < 5 ng/kg	5 – 10 ng/kg	10 – 25 ng/kg	25 – 50 ng/kg	50 – 80 ng/kg	>80 ng/kg
Winter	18.9	20	45.5	15.6	0	0
Spring	21.3	18	47.2	11.2	2.3	0
Summer	17	18	42.3	19.3	2.6	1.05
Fall	78	18	13.5	0	0	0

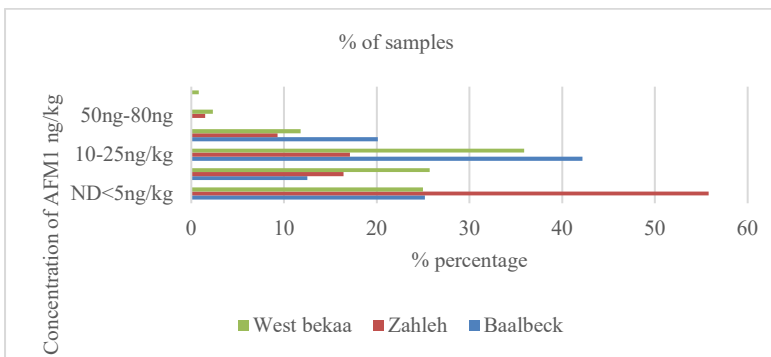

Fig. 12. Percentage of contaminated samples in three region

According to figure 11, it is obvious that during the fall and winter months, contamination levels of the samples from the different farms and regions of Bekaa valley were lower than they were during the summer and spring month. Usually, this could be explained by the prolonged storage required for feed, therefore afla-

toxin M1 contamination of milk is the result of cows feeding on material containing aflatoxin B1; but during the time of collection of fall season samples (October 2021) the temperatures in the Bekaa valley were higher than yearly mean ± SDs, the relative humidity was below mean ± SDs and there were no precipitations, the country was going through a heat wave. Such factors could alter the presence of aflatoxin in feed. The concentration of this mycotoxin in animal feedstuffs is influenced by the type, the time and method of harvesting and temperature and relative humidity of storage facilities (Tajkarimi, *et al.*, 2007), AFM1 concentrations in milk samples (Fig. 12) were significantly higher in the colder seasons (Rahimi *et al.*, 2009). One reason for this is that milking animals are fed with compound feeds in winter that are prone to aflatoxin B1 contamination (Fig. 13).

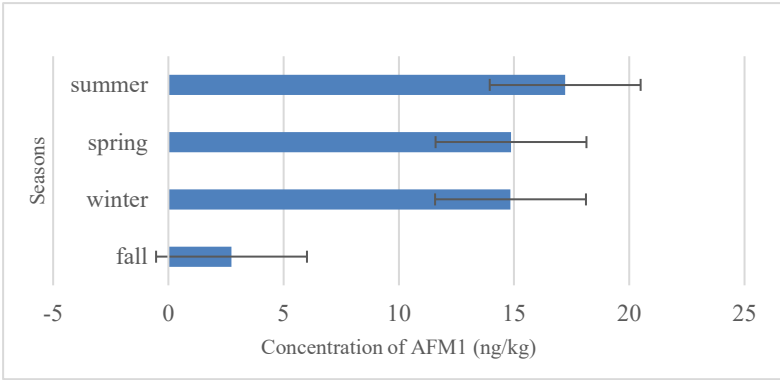

Fig. 13. Concentration of AFM1 (ng/kg) in milk in different seasons

IV.3. EFFECT OF REGIONS AND RAISING SYSTEMS ON MILK AFM1 CONTAMINATION

The mean concentration of AFM1 in milk samples in the three different regions and the four seasons of the study are shown in Table 6. 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 6. 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 7. 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 (Fig. 14).

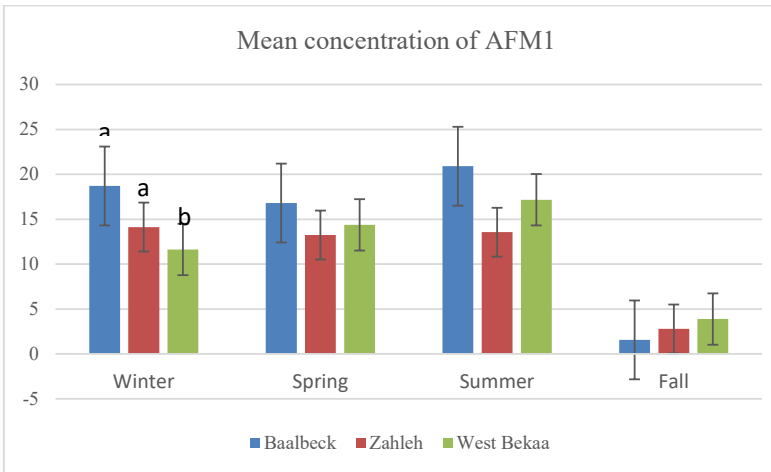

Fig. 14. Mean concentration in of AFM1 (Y axis) in different regions and seasons (X axis)

Table 7. Percentage 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%

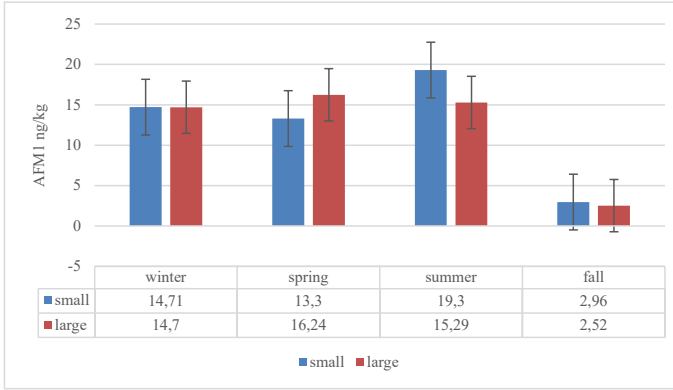

Figure 15 : Incidence of AMF1 during different seasons

According to figure 12 and table 7, 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.

Table 8. 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

As overall the level of aflatoxin M1 contamination recorded in this study was as (Table 8) follow: 1.73% of theanalyzed samples in small scale farms show AFM1 level higher than the European standards. 1.63% of the analyzed milk samples in large scale farms show a level of contami-

nation 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.

IV.4. RESULTS RELATED TO AFB1

After carrying out the inspection of the herds, the results were based on different parameters and criteria including feeding guide and sources of the feed, conditions of the storage site. In addition, presence of factors that impair the physical state of feed (such as bad ventilation and humidity), presence of insects or rodents that could reach the feed storage area, presence of chemical factors that promote growth of fungi in stored feed. If there is an implementation of control measures for pests and rodents, hygiene of the farm and feedlot and presence of molds (on top of corn silage per example). The determined levels of AFB1 in the samples (collected from 15 farms) were variable and found in all samples. One-sample t-test results, minimum, maximum, mean values of AFB1 have been shown in Table 9.

Table 9. Mean levels of AFB_1 (µg/kg) in feedstuffs ($\bar{X}$ ± SD).

Feedstuffs	N	Number of sample above limit of 20 µg/kg	% above limit of 20 µg/kg	$\bar{X}$	SD	Min	Max	*p*-value
Barley bran	15	5	33.33	16.4	7.05	8.30	29.3	0.069
Wheat bran	15	6	40	16.4	9.10	4.32	32.5	0.150
Soybean	15	7	46.67	20.5	15.3	2.56	46.5	0.908
Corn	15	8	53.33	24.7	15.5	5.90	58.5	0.262
Cotton seed	15	9	60	25.1	15.6	5.81	50.3	0.229
Hay	15	9	60	27.0	12.8	10.5	49.6	0.052

N − Number of samples; ($\bar{X}$) − Mean (µg/kg); SD − Standard Deviation; Min − Minimal levels (µg/kg); Max − Maximal levels (µg/kg).

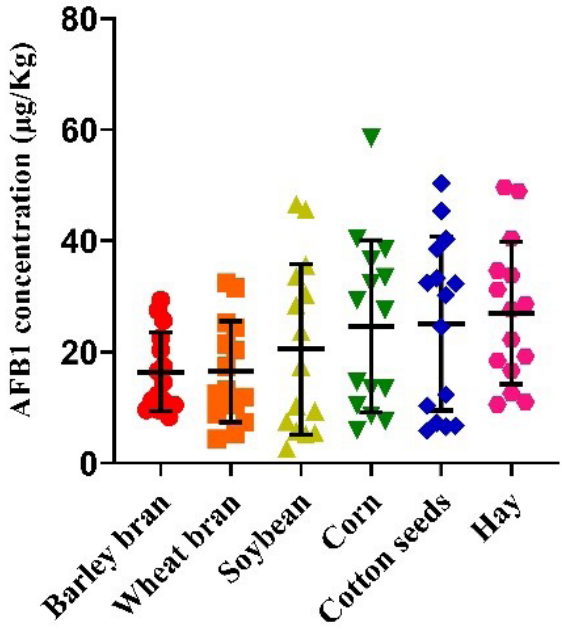

Fig. 16. Distribution of AFB1 levels per feedstuff. The graph shows the mean and SD of AFB1 concentrations (Represented as dots) collected from 15 farms.

Out of 90 samples, 44 samples (48,88%) showed contamination exceeding the limit accepted by USA and Lebanese limit (20 µg/kg).The six type of feedstuffs as cited above: Barley bran, Wheat bran, Soybean, Corn, Cotton seed, and Hay revealed from 33.33 to 60% contamination rate, respectively (Table 9). The maximum level of AFB1 in those samples can be presented in this order: Corn > Cotton seed > Hay > Soybean > Wheat bran > Barley bran. The highest (maximum) level was detected in a sample of Corn, and the lowest (minimum) level was detectd in a sample of Soybean (Table 9, figure 16). The lowest mean of AFB1 was detected in Barley bran and Wheat bran, while in the Hay aflatoxin AFB1 showed the highest mean levels (Table 9). AFB1 mean concentrations in Barley bran and Wheat bran were higher than the European Union legal limit level (5 µg/kg), but lower than the USA and Lebanese legal limit (20 µg/kg). Interestingly, AFB1 mean levels in the samples of Soybean, Corn, Cotton seed, and Hay were higher than the US and Lebanese legal limit (20 µg/kg), but showed no statistical significance when compared to this limit

(Table 9). Mean value of AFB1 levels in Hay were close to be significantly higher than the USA and Lebanese limit. Notably, AFB1 mean concentrations exhibited an upward trend when comparing samples of Soybean, Corn, Cotton seed, and Hay (Fig. 16), but no significant difference was detected among means.

DISCUSSION

After finding out the effects of the aflatoxin on human and animals' health, many countries designated maximum limits of these mycotoxins that exist in foods and fodders. Since having levels under this limit does not mean that is safe, the countries tended to decrease the limits. Aflatoxins are both acutely and chronically toxic for animals and humans and can cause dangerous diseases including acute toxic hepatitis, liver cirrhosis and hepatocarcinoma (Park, 2002; Alborzi *et al*., 2006). AFM1 contamination in dairy products is a global problem threatening public health in all areas of the world. Despite high consumption of dairy products in Lebanon, a few credible data are available on their contamination levels with AFM1.

In our study, out of the all samples analyzed in each districts of Bekaa, only 25.2% were reported free in Baalbeck, 55.8% in Zahleh and 25% in West Bekaa. However, the percentages of milk samples that fail to meet European standards were as follows: 0% in Baalbeck, 1.5% in Zahleh and 3.14% in West Bekaa. The yearly mean ± SD AFM1 contamination in milk samples varies between 14.31 ng/kg in Baalbeck and 11.5 ng/kg in West Bekaa and 10.5 ng/kg in Zahleh. These results are slightly higher than those reported by Hassan and Kassaify (2014) who reported a mean AFM1 content of 10.74 ± 2.01 ng/L in cow raw milk collected from different sites 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 0.005 μg/L to 0.05 μg/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 competitive enzyme – linked immunosorbent assay (ELISA) method was applied for this purpose positively

detecting AFM1 in 64.9% of all tested milk samples. 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. Similar studies conducted in neighboring countries showed also high contamination level.

In Syria, the incidence of contamination of aflatoxin M1 (AFM1) in milk samples collected from the Syrian market was investigated by Ghanem and Orfi (2009) by using the competitive enzyme linked immunosorbent assay (ELISA) technique. 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 aflatoxin M1 in Palestine raw milk collected at farms from Tulkarm, Nablus and Jenin. Aflatoxin M1 was determined by direct competitive ELISA technique. 85 % (34 of 40) of the total examined raw milk samples tested were positive. The aflatoxin M1 contamination levels were between 3 – 80 ppt with a mean of 29.57 ppt. There was a high incidence rate with 92% (11 of 12) and the highest means of contaminated with aflatoxin M1 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. An enzyme –linked immunosorbent assay (ELISA) was used for the analysis of milk samples. AFM1 was detected in all animal milk samples with mean ± SD concentration of 56.17 ng/kg (range 7.05 – 129.79 ng/kg) in fresh milk samples and 1079.57 ng/kg (range 47.97 – 2027.11ng/kg) in fermented milk. 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). A total of 133 liquid milk samples and 137 samples of corresponding feedstuffs collected from 5 local dairy farms in Jordan were tested. In addition, 41 imported powdered milk samples obtained from 3 dairy processing plants were tested. The collected samples were assayed for the level of aflatoxin M_1 in milk and aflatoxin B_1 in feedstuffs. Results of the analysis revealed the presence of aflatoxin M_1 in 5 liquid milk samples with an incidence rate of 3.8% and with toxin levels ranging from traces to 18.7 ppb. Aflatoxin B_1 was found in 3 feed samples with an incidence rate of 2.2%. Toxin levels were 66.0 and 85.2 ppb in two dairy ration samples and 17 ppm in the corn sample. On the other hand, the powdered milk samples were free from any detectable levels of aflatoxin M_1.

In Kuwait, 54 samples of dairy products were analyzed for aflatoxin M1, 28% were ontaminated 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).

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. As feed aflatoxin B1 levels increase, it will be metabolized in the liver and lead to elevated levels of aflatoxin M1 excreted in milk, Therefore, it is important to reduce the occurrence of aflatoxin B1 toxins in feedstuff and take prophylactic measures to prevent factors enhancing toxin production. Management practices in harvest and storage could decrease aflatoxin B1 occurrence in feed.

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 and barley straws, 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. Aflatoxins are highly toxic compounds, it is therefore important to minimize the health risk from AFM1 contamination in milk, which can be consumed by infants and children as the most at-risk groups. Therefore, dairy farmers should be educated on potential health risk of aflatoxins. AFM1 levels should be monitored as a part of quality control procedures in dairy factories. For this reason, milk and other dairy products have to be checked continuously by accurate and precise analytical methods for the presence of AFM1 contamination and quantification. Furthermore, the level of AFB1 in feed can be reduced by monitoring the cultural phases and storage practices for prevention of mould growth and aflatoxin production. On the whole, dairy cattle feed should be kept away from contamination as much as possible. However, if the reduction of animal feed contamination is not practical, the use of highly contaminated feed should be diverted to non-lactating animals.

In other hand, and concerning our results for detection of AFB1, the Aflatoxine contamination in several types of food and feed is inevitable. As a result, much study is conducted to identify this toxin in food and feed (Hell and Mutegi, 2011; Farombi, 2006). In the present study maximum AFB1 concentration in corn samples was higher than the results reported by Scudamore *et al*. (1997) in the United Kingdom (41 µg/kg), and Oruç *et al.* (2006) in Turkey (32.30 µg/kg as total aflatoxin), but lower than Shetty and Bhat (1997) in India (109 µg /kg), and Dawlatana *et al*. (2002) in Bangladesh (245 µg/kg). According to Kaaya and Kyamuhangire (2006), maize samples stocked for more than six months were observed

to have detectable levels of aflatoxin AFB1 more than 20 µg /kg. In this study, the highest level corn (58 µg/kg) was seen in a sample of corn in more than half the farms which were included in the study (53, 33% positive samples). This is due to for long period and bad storage of corn in all majority of the farm. Mean level of AFB_1 with Standard Deviation (SD) for corn (24.7 ± **15.5)** were higher than the USA and Lebanese legal limit (20 µg/kg), and higher than the European Union (5 µg/kg), but showed no statistical significance when compared to this limit. According to Kabak *et al*. (2006), moisture, poor storage conditions, and poor management all contributed to the elevated levels of aflatoxin, observed in competitive cow's feed, feed additives, and maize. In another study conducted in Iraq, AFB1 was found in 12 out of 24 samples of maize, with levels ranging from 2.30 to 30 ppb detectable through thin layer chromatography (TLC) and 270 to 500 ppb by means of Enzyme-Linked Immunosorbent Assay (ELISA) (Hassan *et al*., 2014). It is well known that a variety of conditions, including temperature, humidity, insect damage, handling during harvest, and storage, affect the establishment of *Aspergillus spp*. and the subsequent formation of aflatoxins in maize (Hell *et al*., 2003). Concerning the cotton seeds, in this study 60% of the sample was contaminated and exceeded the limit (20 µg/kg). The mean value and SD of AFB1 concentration was (25.1 ± **15.6** µg/kg), and it was higher more the USA (20 µg/kg) and European Union (5 µg/kg) permissible limit, but no significant difference was detected among means. In comparison with another study from Iran, cotton seeds had highest levels of contamination, even higher than the maximum tolerated levels. All the cotton seed samples (100%) were contaminated by AFB1, which detected by High Performance Liquid Chromatography (HPLC) (Sadegh *et al*., 2013). According to Azizi *et al*. (2012), cotton seeds show a contaminated level of AFB1 compared to concentrated feed and beet pulp. In a study by Hashemi, (2016), the Cotton seeds had an AFB1 level (2.13 ± **0.31** µg /kg) lesser than the present study (25.1 ± 4.03 µg /kg). In the present study, wheat bran samples showed 40% contamination, and they reached a maximum level of AFB_1 at 32.5 µg/kg. The mean ± SD of AFB1 concentration was 16.4 ± **9.10** µg /kg, but no statistically significant difference was detected in the found values. In comparison and According to some authors (Aydin *et al*. (2008); Joubrane *et al*. (2011), and Almeida-Ferreira *et al*., (2013)) have high levels of contamination of wheat and their derivatives with AFB1. In an Indian

study, Aflatoxin B1 (AFB1) levels in 1646 samples of wheat grains were examined. 40.3% of the samples had AFB1 levels below 5 µg/ kg, while 16% had concentrations over 30 µg /kg, allowed regulatory limit for India (Toteja *et al*., 2006). Even though in wheat had a high prevalence of AFB1 (44.8%), wheat samples had the lowest maximum limits of 6.0 µg /kg (Elbashir and Ali, 2014). In a study by Hashemi, (2016), Wheat bran had an aflatoxin AFB1 level (2.94 ± **1.38** µg /kg) lesser than our study (16.4 ± 2.35 µg /kg). In this study, mean levels of AFB1 in Soybean is (20.5 ± **15.3** µg /kg), and were higher than the US and Lebanese legal limit (20 µg/ kg), and higher than EU limit (5 µg/kg), but showed no statistical significance when compared to this limit concentrations. According to Abdullah Murshed *et al*. (2019), 72% of soybean samples were contaminated with aflatoxins, and 27.6% of them exceed the European Standard, and in only 6.2% of the soybean samples taken, the values of total aflatoxins were above the maximum limit by the FDA standards in Yemen (20 µg/kg). In the present study, the highest level of AFB1 was found in the samples of Soybean (46.5 µg/kg), which was higher than that in United Kingdom (4 µg/kg) reported by Scudamore *et al*. (1997), and slightly higher than that in Turkey (46.3 µg/kg) reported by Oruç *et al*. (2007), and higher than the level from Iran (11.46 µg/kg) reported by Hashemi, (2016). Regarding barley bran, 33.33% of the contaminated samples by AFB1 exceeded the USA and Lebanese permissible limit (20 µg/kg). Those results were higher compared with the result of Sadegh *et al.* (2013), who established 0% contamination in barley samples, and higher than the result reported by Hashemi (2016), where barley samples show contamination with AFB1 less than Iran regulations and European Union limitations (5 µg/kg) (1.31 ± 0.33 µg/kg). Regarding Aflatoxin B1 detection in the hay, in our study all samples were found contaminated, and 60% of them were with mean value 27.0 µg/kg, and exceeded the USA and Lebanese permissible limit of 20 µg/Kkg. Our results are higher than those reported by Ceniti *et al*. (2021), which they found in all hay samples levels of AFB1 contamination ranging from 2 to 7.7 µg/kg, which are within the limitations set by the European Union (20 µg/kg). In a study done by Karademir *et al*. (2003), fresh Hay shows average values less than the allowed Turkey limits (50 µg/ kg). Old Hays' average values were below the limit of Turkey (50 µg/kg), but they were above the maximum limits (20 µg/kg). In Northern Italy, Decastelli *et al*. (2007), reported that 8.1% of the feed samples examined

were positive for AFB1. In Iran the concentration of AFB1 in the Hay was higher than the limit of the European Union with 10% (Bahrami *et al*., 2016). Contrarily, some investigations have shown a sizable amount of contaminated samples, rarely reaching the set legal limit. 42% of the samples from China included AFB1 levels between 0.05 and 3.53 µg /kg were below the legal limit in Chinese and European standards (10 µg/kg) (Han *et al*., 2013). In a 10-year investigation in Portugal, Martins *et al*. (2007) discovered 37.4% positive samples with contamination, ranging from 1 to 74 µg/kg, and only 6.2% of these samples surpassed the legal limit of Portuguese (5 µg/kg). Hay samples contamination with mycotoxin in our study, especially with AFB1, is a result of bad storage conditions of the Hay in the farms, e.g., the high percentage of moisture that has not been studied.

All the samples in our study, collected during this period (between the end of June and the start of July), were contaminated with AFB1. Most of the farms from which were collected the samples were small-scale farms from the three regions (R1, R2, R3). The collection of the samples was from the farm, not the original source. In correlation with the observations that we have made about feed management in the different farms, especially storage, all those are in agreement with the results obtained from this study, after testing feed that shows contamination with AFB1 during this period. According to Richards *et al*. (2009), feed storage management and storage conditions facilitated mold development and, as a result, they are the cause of aflatoxin production.

CONCLUSIONS

1. The results of this study revealed high level of aflatoxin M1 contamination in cow milk in different districts of Bekaa Valley. In fact, out of all samples analyzed in each districts, only 25.2% were reported free in Baalbeck, 55.8% in Zahleh and 25% in West Bekaa.

2. 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.

3. The level of aflatoxin M1 was variable according to regions, management practices and season of the year.

4. The highest level of aflatoxin M1 in raw cow milk was recorded in West Bekaa and the lowest level in Baalbeck.

5. Higher level of aflatoxin M1 was also recorded in small holder livestock farms (less than 20 cows) compared to large scale farms.

6. Higher level of aflatoxin M1 contamination in cow milk was recorded in winter, spring and summer compared to fall. In Baalbeck, minimum values were shown in fall and highest in winter in the 2 systems and all samples were lower than EU standards.

7. In Zahleh, 11.1% of samples in spring were above EU standards in small scale farms, 5% at large scale farms and no samples above EU standards in fall, winter and summer. Levels of aflatoxin were surprisingly low during fall season and high during summer.

8. In West Bekaa, zero contamination was recorded in spring and 15.3% in summer in small scale farms. In large scale farms, 2 samples were above EU standards in summer with 0 samples above them in remaining seasons.

9. The mean concentration of milk AFM1 showed a significant difference ($p < 0.05$) in between the different districts of Bekaa Valley the highest values recorded in West Bekaa (>80) and the lowest AFM1 values recorded in milk samples from Baalbeck.

10. The three districts of the study showed a higher AFM1 contamination level in winter, spring and summer the incidence of AFM1 contamination in milk was higher in fall and summer at small scale farms (2.96 and 19.3 respectively), lower in spring (13.3) and equal during winter (14.7).

11. Concerning the concentrations of AFB1 related to different feedstuffs: concentrations are slightly higher than the acceptable limit for cattle consumption and correlate with the occurrence of AFM1 contamination in raw milk that has been studied during this period.

12. From 90 samples of feedstuffs (cotton seed, wheat bran, Barley bran, soybean, hay, and corn) collected mostly from small-scale farms: 44 samples showed contamination by AFB1 and were more than the US and Lebanese Limit (48, 8% of samples).

13. All feedstuffs AFB1 concentrations are higher than US, Lebanese, EU limit but their is no statistical significance when compared to this limit ($p > 0.05$)

14. The highest level seen in corn maize (58 µg/kg) in more than half of the farms and related to bad and long period of storage.

15. At 27 µg/Kg on average and a p-value of 0.052, hay‘s AFB1 levels were very certainly near to the Lebanese and US upper limits and were close to be significant.

16. The findings from our study, particularly regarding the contamination of feed with AFB1, align with our observations on feed management practices across various farms, especially in terms of storage conditions.

RESEARCH CONTRIBUTION:

1. The study is the first to establish the relationship between aflatoxin M1 (AFM1) cow‘s raw milk contamination and farming technology, feeding and seasons in the Bekaa Valley, Lebanon.

2. Regional variations are found in the different districts of the Bekaa Valley. The highest levels AFM1 in cow‘**s** raw milk were detected in the Western Bekaa, while Baalbek had the lowest levels of contamination, indicating geographical differences in contamination.

3. The study have showed seasonal and farm management variations that have impact AFM1 levels. Higher level of milk contamination was found in winter, spring and summer compared to autumn.

4. Small farms (less than 20 cows) showing higher levels of AFM1 in raw milk samples than large farms.

5. The study revealed that Aflatoxin B1 (AFB1) contamination in cattle feed was prevalent, particularly in corn and hay, with levels exceeding the US, Lebanese, and EU limits. Feed contamination correlates with higher levels of AFM1 in milk, leading to a direct link between feed quality and milk safety.

RECOMONDATIONS FOR THE PRACTICE

1. **Establish Rigorous Feed Testing and Management:**
    - Regular testing for AFB1 in animal feed should be mandated, with a focus on locally produced grains and hay.
    - Improved practices like appropriate irrigation, pest control, timely harvesting specially for hay, and safe storage

conditions should be promoted to prevent fungal contamination.

2. **Strengthen Training and Support for Small Dairy Farmers:**
    - Small-scale dairy farms should receive technical assistance and training on contamination prevention, with specific focus on proper feed management, storage, and hygiene.
    - This support should include routine inspections and collection of milk and feed samples to monitor and mitigate contamination risks effectively, especially in remote regions.
    - Farmers in small dairy farms should be informed about the importance of use of mycotoxin binders in the TMR in order to decrease and eliminate the development of mycotoxin
3. **Implement Safe Storage Practices:**
    - Ensure safe storage facilities for animal feed by installing humidity and temperature sensors to maintain optimal conditions.
    - Storage sites should follow good practices, such as regular cleaning and rodent control, to prevent mold growth and toxin production.
    - Continuous monitoring and inspection of these storage facilities by authorities will help maintain compliance.
4. **Enhance Milk Quality Testing at Collection Centers and Cooperatives:**
    - Equip collection centers and cooperatives with the necessary laboratory tools and resources to regularly test milk quality for AFM1 contamination.
    - This will provide an immediate check before milk enters the supply chain, safeguarding product quality.
5. **Conduct Continuous Monitoring and Analysis:**
    - Monitoring AFM1 contamination in milk and dairy products through using accurate and consistent analytical methods that will ensure reliable data on contamination levels and help evaluate consumer exposure to AFM1 across different regions of the Bekaa Valley

6. **Enforce Comprehensive Policies and Regulations:**
    - Political authorities must implement strict regulations and policies focused on reducing AFM1 contamination.
    - Including mandatory testing, defined safety standards for milk and dairy products

Implementing these practices could lead to a reduction in AFB1 and after in AFM1 contamination risks, enhancing the safety of feedstuffs for animals, milk and dairy consumption in Lebanon, especially for children and vulnerable populations.

LIST OF PUBLICATIONS

Conference and Congress papers

Oral presentation at International scientific conference „Tradition and modernity in veterinary medicine“ 2022 – Undola, Bulgaria:

1. Majd Abi Haidar, Charbel Kiwan, Mona Abboud, Toni Todorov, 2022. A review of aflatoxin M1 in raw milk: importance on human health and ruminants, Tradition and Modernity in Veterinary Medicine, vol.7, No 2(13): 143-153, ISSN 2534-9333.

Oral presentation and poster at Congress “JNGTV: Journees nationales des groupemeents techniques veterinaires“ 15-17 May, 2024 - Tours, France:

2. Majd Abi Haidar, Toni Todorov, Mona Abboud, 2024. Prevalence et détection des types d’Aflatoxines dans le lait cru des vaches laitières élevées dans différents systèmes d’élevage, nutrition et saison dans la Vallée de la Bekaa, Liban. SNGTV Journees des GTV Actualites en Sante Animale, p. 36, ISSN 3003-4221.

Journal papers

3. Majd Abi Haidar, 2023, Prevalence and detection of aflatoxin M1 in dairy cow raw milk raised under different breeding systems, nutrition and season in Bekaa Valley- Lebanon, Tradition and Modernity in Veterinary Medicine, vol.8, No 2(15): 3-11, ISSN 2534-9333, e-ISSN 2534-9341. https://doi.org/10.5281/zenodo.7707633.

4. Majd Abi Haidar, Mona Abboud, Toni Todorov, 2023, Detection of aflatoxin B1 in different animal feedstuffs in three regions of Bekaa Valley–Lebanon, Tradition and Modernity in Veterinary Medicine, vol.8, No 2(15): 12-18, ISSN 2534-9333, e-ISSN 2534-9341. https://doi.org/10.5281/zenodo.1033

ABSTRACT

Aflatoxins are both acutely and chronically toxic for animals and humans and can cause dangerous diseases including acute toxic hepatitis, liver cirrhosis and hepatocarcinoma. AFM1 contamination in dairy products is a global problem threatening public health in all areas of the world. Despite high consumption of dairy products in Lebanon, a few credible data is available on their contamination levels with AFM1.The aim of this study was to identify the level of aflatoxin M1 in cow raw milk produced in three different dairy regions in Bekaa Valley, Lebanon (Baalbek, Zahleh and West Bekaa) characterized by different farming types and by different microclimates over one year. Moreover, this study was conducted under the usual and normal conditions of herds' management rather than under defined and controlled experimental conditions. A total of 352 raw milk samples were collected and analyzed from the farms of the three regions of Bekaa Valley from October 2021 to August 2022. The quantity of aflatoxin M1 was determined according to Enzyme-linked Immuno Sorbent Assay (ELISA). Only 25.2% were reported free in Baalbeck, 55.8% in Zahleh and 25% in West Bekaa. The yearly mean ±SD AFM1 contamination in milk samples varies between 14.31 ng/kg in Baalbeck and 11.5 ng/kg in West Bekaa and 10.5 ng/kg in Zahleh. The highest level of aflatoxin M1 was also recorded in small holder livestock farms (less than 20 cows) compared to large scale farms. Finally, higher level of aflatoxin M1 contamination in cow milk was recorded winter, spring and summer compared to fall. A significant difference was seen in winter between seasons (p value 0.032) and in West Bekaa between regions (p value 0.012). In other hand and to show the relation with result obtained from milk samples and detection of AFM1, another study have aimed to determine the levels of AFB1 in different animals'feedstuffs (Soybean, Corn, Cotton seeds, Barley bran, Wheat bran and Hay). For this purpose 90 samples were collected from 15 small farms of three regions of the Bekaa Valley Baalbeck (R1), Zahleh (R2) and West Bekaa (R3) between June 2022 and July 2022. Samples were placed in plastic bags and transported to the laboratory within 12 hours. Samples were stored at 4°C and analyzed for AFB1 within seven days after collection using a competitive Enzyme-Linked Immunosorbent Assay (ELISA) technique. Mean of AFB1 levels were exceeded the maximum for European Union accepted levels for all

samples and USA and Lebanese permissible levels for Soybean, Cotton seed, Corn and Hay but showed no statistical significance when compared to this limit (p>0.05). This finding shows that feed producers and dairy farmers should maintain sustainable good procedures for all feed harvesting, storage, and feeding techniques in order to avoid aflatoxin contamination in feedstuffs by AFB1 and after contamination of raw milk by AFM1.

Keywords: Aflatoxin; AFB1; AFM1; ELISA; Dairy farm; Feed; Raw milk.