

Opposite Direction for Seasonal Variation of Aflatoxin M1 in Bulk Milk and Aflatoxin B1 in Rations: Results From a Prospective Study in Selected Dairy Farms of Qazvin Province, Iran

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Abstract

The presence of aflatoxin M₁ (AFM₁) in 24h bulk milk and aflatoxin B₁ (AFB₁) in concurrent total mixed rations (TMR) and feed ingredients were assessed in 12 large dairy operations. The bulk milk was sampled on days 1, 15 and 30 during winter and summer (n=72). Total mixed rations (n=48) and feed ingredients (n=230) were sampled two times with a 30-day interval. Aflatoxin was measured using direct competitive ELISA kits with detection limits of 1-81 ngkg⁻¹ for milk and 1.25-101.25 ngkg⁻¹ for feeds. Aflatoxin M₁ was identified in all milk samples (100%), ranging from 2.03 to >81 ngkg⁻¹, with a median of 70 ngkg⁻¹ and averaging 61.25±28.91 ngkg⁻¹ in winter and 54.20±25.51 ngkg⁻¹ in summer (P=0.279). Contaminations <81 ngkg⁻¹ (below the Iranian standard of 100 ngkg⁻¹) were detected in 76% (n=55/72) of samples. Contaminations >81 ngkg⁻¹ were detected in 24% (n=17/72) of samples and were more frequent in winter than in summer (42% vs. 6%). Sixty-nine percent of the winter milk samples (n=25/36) had contaminations above the median (70 ngkg⁻¹). A reverse result was detected in summer. The chance of contaminations above median was higher in winter than in summer (OR=5.33, P=0.007). All TMR and ingredient samples had higher AFB₁ contaminations in summer (P<0.05). Six TMR samples had non-detectable (<1.25 ngkg⁻¹) values (5 in winter) and 7 samples had levels >101.25 ngkg⁻¹ (all in summer). The chance of TMR contamination above the median (716 ngkg⁻¹) was 5.57 times higher in summer than in winter (P=0.002). Seventy percent of the TMR samples had contaminations above the median in summer. Elevated levels of AFB₁ of rations in summer (1375.50±905.02 vs. 537.05±558.79; P<0.002) did not result in elevation of AFM₁ in milk, probably due to reduced AFB₁ metabolism in the liver and lower dry matter intakes caused by heat stress. The AFB₁ content of grain mix succeeded by corn silage, wet beet pulp, dry beet pulp and alfalfa hay were correlated with TMR contamination. Ration AFB₁ and milk AFM₁ were not correlated. Based on the results, a great majority of milk produced in the studied farms could have AFM₁ contaminations below the Iranian standard limit (100 ngkg⁻¹). Contaminations below 50 ngkg⁻¹ appear to be achievable and affordable. Intensifying the controlling measures in summer, when the feed contaminations are elevated, may reduce the overall milk contamination.

Introduction

Aflatoxin M₁ (AFM₁), a resistant immunosuppressive and carcinogenic toxin for humans, is secreted into milk after hepatic bio-transformation of dietary aflatoxin B₁ (AFB₁) (Gallo et al., 2015; Alvarado et al., 2017). Many types of feeds, including common feeds of dairy cows, may be contaminated to AFB₁ (Alvarado et al., 2017; Bahrami et al., 2016; Kocasari et al., 2013; Sahin et al., 2013, 2016; Zheng et al., 2013). Widespread contamination of feedstuffs by aflatoxins necessitates some regular actions in dairy farms for hazard analysis and defining the critical control points. Aflatoxin regulatory limits vary between countries based on their specific considerations. The most proposed limits for AFM₁ in raw milk are 50 ngkg⁻¹ (as low as reasonably achievable) and 500 ngkg⁻¹ (regards to the carcinogenic effects of AFM₁) (FAO/WHO, 2002; 2001). It has been stated in some FAO/WHO reports (2001; 2002) that 500 ngkg⁻¹ AFM₁ may be high because any level of genotoxic carcinogens might pose a health risk to consumers. However, it has also been noted that the limit of 50 ngkg⁻¹ may not be achievable in some regions of the world and the limit of 500 ngkg⁻¹ is adequate for the protection of consumer health. To achieve the strict limit of 50 ngkg⁻¹, the maximum allowable concentrations of AFB₁ in dairy feeds and rations is 5,000 ngkg⁻¹ (European Commission, 2011; Kunsagi et al., 2011; Alvarado et al., 2017). The current Iranian standards for AFM₁ and AFB₁ are 100 ngkg⁻¹ and 5,000 ngkg⁻¹, respectively (ISIRI, 2016; 2020).

Recent Iranian surveys and meta-analyses (Mashak et al., 2016; Tajik et al., 2016; Hamzeh Pour et al., 2020; Khaneghahi-Abyaneh, et al., 2019) have shown that the level of AFM₁ in raw and pasteurized milk may be well below the ISIRI limit (100 ngkg⁻¹) but may exceed the limit of 50 ngkg⁻¹, and few samples may exceed the limit of 500 ngkg⁻¹. Also, some studies have assessed AFB₁ contamination of feeds including corn silage, alfalfa hay, straw, barley, wheat bran, beet pulp, canola meal and grain mix and have reported various prevalence of contamination (19.2%: Beheshti and Asadi, 2014; 82.5%: Bahrami et al., 2016; 100%: Rezaei et al., 2014). In the study of Bahrami et al. (2016), 65% of corn silage and 10% of straw samples had contaminations above 5,000 ngkg⁻¹. Seasonal differences in the levels of AFM₁ (Khaneghahi-Abyaneh, et al., 2019; Mozaffari Nejad et al., 2019; De Roma et al., 2017; Mahmoudi and Norian, 2015; Bahrami et al., 2016; Heshmati and Milani, 2010; Kamkar, 2005) and AFB₁ in feeds (Bahrami et al., 2016; Dimitrieska-Stojković et al., 2015; Mahmoudi and Norian, 2015; Simas et al., 2007) have been reported with higher levels in cold seasons.

The AFM₁ contamination of milk and its seasonal variations would be best controlled if the level of AFB₁ in the concurrent rations and feed ingredients are defined. In the present study, performed in a number of large dairy operations during cold and warm seasons, the level and the seasonal changes of AFM₁ were screened in bulk milk to assess the probable hazard. The AFB₁ contaminations of the concurrent rations and separate feed ingredients were also assessed to explain the most critical dietary contaminants in the studied farms.

Materials And Methods

Farms and samplings

The study was done in twelve selected dairy farms in Qazvin province, north-west, Iran, during winter and summer, 2019. The criteria for selecting the farms were having distinct role in the provision of the total milk produced in the province, being scattered in various parts of the province, willing for cooperation and provision of the requested data. The farms owned about 17,700 milking cows during the study period and produced about 40% of the total daily raw milk in the province (Cooperative Organization of Dairy Farms, Qazvin province; personal communication). The average milk production of the farms within the year of study was 30.5 kg/cow/day. In each season, in a 30-day period, bulk milk and rations were sampled. The bulk milk produced within the last 24 hours was sampled on days 1, 15 and 30 (n=36/season, 100ml in screw tap sterile bottles). Samples were taken according to the procedure No 87-44-05 communicated by Iranian Veterinary Organization (IVO, 2005) from a depth of about 20cm of the top surface in the milk tank. The stirrers were working for about 10 minutes before sampling. The samples were transferred cool to the laboratory and were kept frozen (-19°C) for about 30 days before the measurement of AFM₁.

Sampling of feeds was performed 2 days before the first and the last bulk milk sampling. The total mixed rations (TMR) of the milking cows were sampled two times (30 days apart) from the mangers immediately after the distribution of the morning meal according to the procedure described by Robinson and Meyer (2010) (n=24/season; about 0.5kg each). The ingredients of the rations (Table 1) were also sampled (n=230, both seasons; about 0.5kg each) in zipped nylon bags (Procedure No 84-44-06; IVO, 2008). The wet samples (TMRs, corn silage and wet beet pulp) were transferred cool to the laboratory and were oven dried at 50 to 70°C (24 hours; Memmert, UM200, Germany). The samples were powdered in a mill carefully cleaned for each sample, were heated at 105°C for 24 hours and AFB₁ was measured in dry matter. Completion of sampling, transferring and collecting the required data (e.g. the formulations of the rations) all were performed by one trained person and took about two months in each season. Storing and preparation of the samples and measurement of AFM₁ and AFB₁ on milk and feed samples were done at the central diagnostic laboratory of the Veterinary Organization, Qazvin, Iran.

Measurement of AFM₁ in milk and AFB₁ in feeds

The concentration of AFM₁ in milk and AFB₁ in feeds were determined using direct competitive ELISA kits (Bioassay Technology Laboratory, China). The kits were stored at 2-8°C, and before using they were put at room temperature (25±2°C) for two hours. All steps of the experiments were done avoiding direct sun light.

AFM₁

The frozen milk samples were thawed at room temperature (25±2°C) and after reaching to thermal equilibrium, they were centrifuged at 3000g for 10 minutes. The skimmed milk was used for AFM₁ determination. Ten ml of each sample was mixed with 20 ml of 70% ethanol in a screw-tap vial and was shaken vigorously for 3 minutes. Then, the mixtures were centrifuged at 3000g and 100 µL of the supernatant liquid was mixed with 400 µL of the diluent solution. Using the provided microplate, 50 µL of the standard solutions (0.1, 0.3, 0.9, 2.7 and 8.1 ppb of AFM₁) were put in the corresponding wells for preparing the standard curve. The same amounts of the prepared samples were put in the other wells. To all wells, was added 50 µL of anti-AFM₁ conjugate antibody. The plate was shaken gently for a few seconds and then was warmed in 37°C bath for 30 minutes. The liquid in the holes was emptied and the microplate was washed 5 times (Bio-Tek ELx50, Bio-Tek Instruments, USA) using the washing solution. To all holes, were added 50µL of color solutions A and B and the plate was put in 37°C bath for 10 minutes (formation of blue color). Finally, 50µL of the stop solution was added to each hole to terminate the reaction (formation of yellow color). The absorbance of the samples was determined at 450nm within 5 minutes (Bio-Tek ELx800, Bio-Tek Instruments, USA). The absorbance value was inversely related to the concentration of AFM₁. The analytical range of the AFM₁ kit was 1-81 ngkg⁻¹. The values were reported as ngkg⁻¹ (ppb).

AFB₁

Five grams of each powdered feed sample was mixed with 25 ml of 70% ethanol in a screw-tap vial, was left at room temperature overnight and then was shaken vigorously for 10 minutes. Then, the samples were centrifuged at 3000g and the supernatant liquids were filtered on Whatman 1 papers. Exactly 100 µL of the filtered liquid was mixed with 400 µL of the diluent solution. The other steps for measuring AFB₁ were the same as described for AFM₁. The standard curves were prepared using the standard solutions of 0, 0.05, 0.15, 0.45, 1.35 and 4.05 ppb of AFB₁. The analytical range of the AFB₁ kit was 1.25-101.25 ngkg⁻¹. The values were reported as ngkg⁻¹ (ppb). The AFB₁ concentrations in TMRs (Table 1) were reported as measures calculated from the weights and AFB₁ contaminations of separate ingredients (calculated TMR) and also as the contaminations of the samples taken from the bunks (bunk TMR).

Statistical analysis

The results were statistically studied using SPSS software (version 24, Illinois, USA). For aflatoxin values out of the analytical range of the corresponding kit (1-81 ngkg⁻¹ for AFM₁; 1.25-10.25 ngkg⁻¹ for AFB₁) the lower and the upper limits were substituted as the lowest and the highest detected values, respectively. Descriptive statistics were presented as means and standard deviations. The aflatoxin levels in milk and bunk TMR samples were classified into two relatively equal-sized groups based on their medians. Association of the aflatoxin with season was evaluated using generalized estimating equations (GEE) with binomial distribution and logit function. Farm was introduced into the model as subject effect and season as well as number of samplings in each season were considered as repeated effects to account for the dependence between measurements. Separate models were constructed for AFM₁ and AFB₁ in milk and bunk TMR, respectively. The aflatoxin concentration in the calculated TMR and ration ingredients as well as ration DM (bunk and calculated, see below) of winter and summer were compared using linear mixed models. Farm was considered as random effect, and season and number of samples as fixed effects in the model. For ration ingredients, an additional fixed effect for type of feeds was introduced into the model followed by multiple comparisons with Bonferroni adjustment. The relationships between milk AFM₁ with AFB₁ in the TMR (bunk and calculated) and the separate ration ingredients were assessed by Spearman's rho correlation test. The P-value less than 0.05 was considered significant.

Table 1

Weight, AFB₁ concentration and the contribution of ration ingredients in total contamination of the ration

Farm	Ration ingredient	Feed weight in the ration (kg DM)				Feed AFB ₁ (ng/kg DM)				Total AFB ₁ (ng) in the whole ration DM			
		Winter		Summer		Winter		Summer		Winter		Summer	
		2	1	2	1	2	1	2	1	2	1	2	
1	Alf. hay	2.09	2.07	4.46	4.24	30.69	78.95	35.31	4.84	64.17	163.26	157.41	20.53
	Corn sil.	13.15	4.97	3.80	4.33	3.03	1.94	36.15	10.08	39.84	9.63	137.51	43.61
	BP-dry	-	-	-	-	12.58	7.35	16.42	7.98	-	-	-	-
	BP-wet	2.37	1.17	-	1.39	2.37	1.25	-	101.25	5.61	1.46	-	140.71
	Grain mix	13.19	13.28	12.71	12.67	2.39	1.25	27.38	101.25	31.53	16.60	347.90	1283.26
	TMR (calculated)	30.8	21.49	20.97	22.64					141.14	190.96	642.86	1488.11
TMR (bunk)	14.17	21.84	21.22	22.11	1.25	24.35	52.49	101.25	17.72	531.84	1113.62	2238.80	
2	Alf. hay	2.76	2.71	2.83	2.85	1.87	101.25	101.25	101.25	5.16	274.04	287.01	288.48
	Corn sil.	4.37	5.89	4.73	4.77	1.25	42.27	67.79	23.52	5.46	248.85	320.35	112.13
	BP-dry	0.64	0.63	0.92	0.75	18.88	19.49	101.25	1.25	12.11	12.34	93.56	0.94
	BP-wet	-	-	-	-	2.00	91.98	-	5.48	-	-	-	-
	Grain mix	15.18	15.50	15.40	15.61	12.68	4.77	65.12	16.36	192.44	73.95	1002.83	255.45
	TMR (calculated)	22.95	24.73	23.88	23.98					215.23	609.19	1703.67	656.99
TMR (bunk)	32.79	20.36	18.51	14.91	28.63	34.39	240.67	81.20	938.19	700.09	1874.22	1210.67	
3	Alf. hay	1.84	1.86	2.60	2.58	6.11	24.71	101.25	13.48	11.27	45.95	263.07	34.81
	Corn sil.	7.74	9.30	6.68	4.47	20.69	1.25	92.84	101.25	160.08	11.63	620.40	452.44
	BP-dry	1.35	1.38	0.94	0.95	14.77	27.29	10.42	9.52	19.91	37.78	9.81	9
	BP-wet	-	-	-	-	15.23	48.45	21.86	91.42	-	-	-	-
	Grain mix	15.38	15.48	14.08	13.71	94.80	62.77	27.22	7.34	1457.69	971.37	390.39	100.61
	TMR (calculated)	26.31	28.02	24.31	21.70					1648.95	1066.73	1283.68	596.86
TMR (bunk)	27.64	26.80	20.04	22.84	2.73	5.23	10.51	13.29	75.47	140.15	210.63	303.57	
4	Alf. hay	1.81	1.83	2.98	3.04	17.20	101.25	101.25	11.63	31.21	184.82	301.26	35.37
	Corn sil.	5.91	4.35	4.81	5.03	1.99	53.42	50.33	101.25	11.75	232.46	242.20	508.92
	BP-dry	1.82	1.84	-	-	1.83	49.23	-	101.25	3.34	90.41	-	-
	BP-wet	-	-	-	-	-	-	-	-	-	-	-	-
	Grain mix	15.33	15.33	14.32	14.55	24.84	13.05	52.49	101.25	380.85	202.62	751.76	1473.17
	TMR (calculated)	24.87	23.35	22.11	22.62					727.16	710.31	1295.21	2017.47
TMR (bunk)	39.45	19.59	19.03	22.15	33.24	3.93	99.33	101.25	1311.15	76.97	1889.81	2243.09	
5	Alf. hay	1.82	1.87	1.82	1.90	21.28	101.25	92.84	101.25	38.83	188.85	169.04	192.27
	Corn sil.	11.12	6.94	9.13	6.17	15.79	4.34	51.94	6.25	175.64	30.10	474.30	38.56
	BP-dry	0.55	0.56	0.55	0.59	1.39	21.07	57.04	75.71	0.77	11.78	31.43	44.68
	BP-wet	-	-	-	-	3.12	1.25	80.22	9.69	-	-	-	-
	Grain mix	15.42	15.49	15.41	15.25	21.15	7.10	68.47	5.20	326.08	110.01	1055.39	79.29
	TMR (calculated)	28.91	24.86	26.91	23.91					541.32	340.74	1730.16	354.81
TMR (bunk)	30.26	32.34	22.28	24.77	2.74	22.63	70.56	69.87	82.91	731.89	1572.38	1730.56	
6	Alf. hay	4.11	4.17	4.49	4.53	4.34	16.01	20.06	101.25	17.86	66.69	90.14	459.03
	Corn sil.	4.69	8.11	4.80	4.58	1.25	5.05	43.76	101.25	5.86	40.97	209.84	463.64
	BP-dry	-	-	-	-	6.40	22.31	9.43	6.93	-	-	-	-

Table 1

Weight, AFB₁ concentration and the contribution of ration ingredients in total contamination of the ration

	BP-wet	1.01	0.99	1.19	1.02	1.25	1.54	97.43	5.30	1.27	1.53	115.92	5.40
	Grain mix	13.18	13.39	15.44	15.38	1.77	4.96	46.20	13.69	23.33	66.40	713.44	210.56
	TMR (calculated)	22.99	26.66	25.92	25.51					48.32	175.59	1129.34	1138.64
	TMR (bunk)	33.08	32.60	27.36	25.20	18.58	50.04	1.25	101.25	614.58	1631.05	34.19	2551.20
7	Alf. hay	1.83	1.86	1.65	1.64	101.25	3.22	1.25	101.25	187.75	5.99	2.06	166.05
	Corn sil.	5.46	7.13	4.86	4.65	16.54	5.08	57.63	45.34	90.32	36.20	279.94	210.77
	BP-dry	-	-	-	-	1.25	20.63	40.96	9.77	-	-	-	-
	BP-wet	1.01	1.08	0.81	1.27	16.91	28.88	46.20	7.01	17.06	31.19	37.38	8.89
	Grain mix	15.39	15.69	15.47	15.49	34.16	11.06	8.70	101.25	325.55	173.49	134.57	1568.06
	TMR (calculated)	23.69	25.75	22.79	23.05					818.68	246.86	453.96	1953.76
	TMR (bunk)	26.22	29.75	17.89	19.85	25.13	1.25	58.83	101.25	658.91	37.18	1052.70	2009.71
8	Alf. hay	1.14	1.16	1.89	1.82	1.25	60.84	84.25	101.25	1.43	70.70	159.54	184.05
	Corn sil.	9.39	6.40	7.01	7.18	4.56	25.08	70.56	7.16	42.81	160.40	494.80	51.41
	BP-dry	-	-	-	-	2.24	2.89	101.25	85.12	-	-	-	-
	BP-wet	1.09	1.03	1.22	1.44	3.16	2.21	56.46	20.87	3.45	2.27	68.65	30.05
	Grain mix	13.74	14.08	16.02	16.02	1.25	4.81	33.69	60.08	17.18	67.73	539.81	962.66
	TMR (calculated)	25.36	22.67	26.14	26.46					64.87	301.08	1262.80	1228.18
	TMR (bunk)	33.23	25.62	25.72	23.21	22.70	1.25	83.42	99.82	754.29	32.02	2145.75	2316.74
9	Alf. hay	2.40	2.37	2.32	2.36	16.07	79.86	98.37	101.25	38.58	189.20	228.46	238.87
	Corn sil.	7.96	5.08	4.95	5.63	2.47	1.25	5.72	3.56	19.66	6.36	28.30	20.04
	BP-dry	0.92	0.91	0.90	0.93	4.11	11.05	28.42	1.25	3.76	10.03	25.66	1.17
	BP-wet	-	-	-	-	-	-	-	-	-	-	-	-
	Grain mix	13.15	13.23	12.68	12.70	5.46	5.01	40.50	8.68	71.82	66.30	513.42	110.26
	TMR (calculated)	24.43	21.59	20.85	21.62					133.82	271.87	795.85	370.33
	TMR (bunk)	25.26	15.85	20.47	19.15	17.78	1.25	2.98	5.83	449.18	19.81	61.01	111.63
10	Alf. hay					2.35	101.25	101.25	20.39				
	Corn sil.					4.49	21.99	74.15	78.48				
	BP-dry					3.95	45.51	89.31	9.94				
	BP-wet					2.46	1.25	71.97	86.40				
	Grain mix					1.25	16.50	65.12	101.25				
	TMR (calculated)												
	TMR (bunk)					3.92	1.25	96.49	101.25				
11	Alf. hay					101.25	1.25	1.25	101.25				
	Corn sil.					22.80	57.99	101.25	101.25				
	BP-dry					2.76	20.19	101.25	2.23				
	BP-wet					2.33	29.37	101.25	25.74				
	Grain mix					4.16	10.92	35.73	8.06				
	TMR (calculated)												
	TMR (bunk)					23.73	71.47	81.81	101.25				
12	Alf. hay	3.13	3.26	2.80	2.85	25.42	35.90	83.42	1.25	79.65	117.11	233.57	3.56
	Corn sil.	4.37	10.13	4.69	3.45	14.95	30.67	35.31	14.02	65.30	310.63	165.53	48.31

	BP-dry	2.02	2.03	2.26	2.31	1.25	16.37	27.38	97.46	2.53	33.22	61.94	225.25
	BP-wet	-	-	-	-	1.25	29.88	1.25	101.25	-	-	-	-
	Grain mix	15.39	15.64	15.33	13	22.33	11.06	1.25	45.35	343.66	172.94	19.17	589.62
	TMR (calculated)	24.91	31.06	25.08	21.61					491.14	633.90	480.21	866.75
	TMR (bunk)	22.19	32.82	16.43	24.59	2.95	57.05	21.33	101.25	65.46	1872.10	350.45	2489.60

Alf. hay: alfalfa hay; corn sil.: corn silage; BP: beet pulp

Results

Aflatoxin M₁ in milk

Aflatoxin M₁ was detected in all bulk milk samples (n=72; 100%) ranging from 2.03 to >81 ngkg⁻¹ (Table 2). The average seasonal contaminations (considering 81 ngkg⁻¹ as the highest detected value) were 61.25±28.91 ngkg⁻¹ in winter and 54.20±25.51 ngkg⁻¹ in summer (P=0.279); and the median was 70 ngkg⁻¹. The frequencies of the samples with AFM₁ levels below and above the median are shown in Table 2.

Farms	Winter				Summer			
	1	2	3	Mean	1	2	3	Mean
1	5.13	74.89	4.74	28.16	79.59	67.41	27.86	58.29
2	69.40	>81.00	>81.00	>77.13	45.18	31.97	67.41	48.19
3	51.45	20.50	12.51	28.15	67.00	74.61	80.52	74.04
4	>81.00	>81.00	>81.00	>81.00	4.51	47.97	>81.00	>44.49
5	80.81	71.25	70.02	74.03	70.31	1.74	77.30	49.78
6	8.73	>81.00	>81.00	>56.91	>81.00	17.38	2.03	>33.47
7	>81.00	70.02	79.64	76.89	4.09	22.15	69.89	32.04
8	5.88	73.69	72.47	50.68	70.31	65.79	60.75	65.62
9	>81.00	>81.00	>81.00	>81.00	56.71	43.39	68.23	56.11
10	>81.00	>81.00	14.47	>58.82	25.73	69.47	79.59	58.26
11	4.98	>81.00	>81.00	>55.66	63.43	52.54	49.89	55.29
12	56.38	71.87	70.02	66.09	75.06	79.95	69.47	74.83
Frequencies below and above the median								
	Sum				Sum			
<70 ngkg ⁻¹	7	1	3	11	7	10	8	25
≥70 ngkg ⁻¹	5	11	9	25	5	2	4	11

Figure 1 shows that 29% of samples (21 out of 72) had AFM₁ levels below 50 ngkg⁻¹, 47% (34 samples) had contaminations between 50-81 ngkg⁻¹ and 24% (17 samples) had contaminations above 81 ngkg⁻¹. The contamination of the latter category could potentially be below or above the Iranian standard (100 ngkg⁻¹). Overall, 76% of bulk milk samples (56 out of 72) had aflatoxin levels less than 81 ngkg⁻¹.

Regarding the seasonal variations, contaminations >81 ngkg⁻¹ were more frequent in winter (42% in winter vs. 6% in summer; Figure 2). Examining the median AFM₁ contamination, 69% of winter samples (n=25/36) had contaminations above 70 ngkg⁻¹ and a reverse result was detected in summer (Table 2). In fact, the results of GEE for association of milk aflatoxin with season showed the chance of contaminations above 70 ngkg⁻¹ was 5.33 times higher in winter than in summer (OR=5.33, P=0.007).

Aflatoxin B₁ in Feed ingredients and TMRs:

The weights (DM), AFB₁ contamination and the share of each ingredient in the whole ration contamination, and total AFB₁ content of the TMR are shown in Table 1. The data on TMR were calculated from the weight and AFB₁ contamination of separate ingredients (calculated TMR). The contamination of the TMR samples taken from the bunks are shown in separate rows (bunk TMR). Two farms did not provide the ration details and so, only the contamination of their feed samples is shown.

The average AFB₁ contaminations of separate ration ingredients (n=230) and the frequencies of non-detectable (<1.25 ngkg⁻¹) and high (>101.25 ngkg⁻¹) contaminations are depicted in Table 3. All samples had significantly higher contaminations in summer compared with winter. Alfalfa hay had the highest averages in both seasons (P<0.05). It also showed the most frequent values above 101.25 ngkg⁻¹ DM. Twenty-three samples (10%) had non-detectable levels of AFB₁ and 32 samples (13.9%) had values above 101.25 ngkg⁻¹. The non-detectable values were mostly seen in winter (n=17/23), while the values above 101.25 ngkg⁻¹ were mainly detected in summer (n=27/32).

Table 3								
Aflatoxin concentration (mean±SD) ¹ in the ration ingredients in the studied farms								
Feeds	Number of Samples		Aflatoxin concentration (ng/kg DM)		Mean	Frequencies		
	Winter	Summer	Winter	Summer		<1.25	>101.25	
Ingredients								
Alfalfa hay	24	24	39.80±38.92 ^a	65.92±43.36 ^{a*}	52.86±42.85 ^a	5	16	
Corn silage	24	24	15.01±16.79 ^b	53.37±35.40 ^{b*}	34.19±33.59 ^b	4	5	
BP-dry	24	23	13.94±13.19 ^b	43.11±40.49 ^{b*}	28.22±32.99 ^b	4	4	
BP-wet	20	19	14.31±22.80 ^b	51.73±39.58 ^{b*}	32.03±36.68 ^b	6	3	
Grain mix	24	24	15.81±21.70 ^b	43.42±33.49 ^{b*}	29.62±31.21 ^b	4	4	
Sum	116	114				23	32	
1: assuming that the values of 1.25 and 101.25ngkg ⁻¹ (the lower and the upper limits of the experimental kit) were the lowest and the highest contamination rates; a, b: different letters indicate to significant differences in the columns (P<0.05), *: Asterix indicate to significant differences in rows (P<0.05); BP: beet pulp; DM: dry matter								

The average AFB₁ content of the TMRs (Table 4) was higher in summer than in winter for both calculated (P<0.001) and bunk (P=0.002) TMRs, so that each cow could potentially consume at least 2.4-2.6 times more aflatoxin in summer. The bunk samples were contaminated higher than the calculated TMRs. Among the bunk TMRs (n=48), 6 samples had non-detectable values (5 in winter) and 7 samples had levels above 101.25 ngkg⁻¹ (all in summer; Table 4). The median contamination of bunk TMRs was 716 ngkg⁻¹ and the GEE results showed that the chance of contaminations above median was 5.57 times higher in summer than in winter (OR=5.57, P=0.002). Seventy percent of the bunk TMR samples had contaminations above the median in summer (Figure 3).

Table 4								
The concentration (ngkg ⁻¹ DM) and the total daily content (ng) of AFB ₁ (mean±SD)* in TMRs of the studied farms								
Season and sampling						Averages		
TMR type	Winter1	Winter2	Summer1	Summer2	Min	Max	Winter	Summer
Calculated								
Ration DM (kg)	25.53±2.54 (n=10)	25.04±3.00 (n=10)	23.90±2.16 (n=10)	23.31±1.66 (n=10)	20.85	31.06	25.28 ±2.72	23.60 ±1.90
							P=0.017	
AFB ₁ (ng/kg DM)	17.69±18.79 ^a (n=10)	17.80±10.30 ^a (n=10)	44.68±17.86 ^b (n=10)	44.78±26.44 ^b (n=10)	2.10	89.20	17.74 ±14.74	45.22 ±21.97
							P<0.001	
Daily AFB ₁ intake (ng)	453.06±488.08 ^a (n=10)	454.72±289.94 ^a (n=10)	1077.80±466.52 ^b (n=10)	1067.20±607.36 ^b (n=10)	48.32	2017.47	453.89 ±390.72	1072.50±527.12
							P<0.001	
Bunk								
Ration DM (kg)	28.43±7.04 (n=10)	25.76±6.13 (n=10)	20.90±3.43 (n=10)	21.88±3.16 (n=10)	14.17	39.45	27.09 ±6.57	21.39 ±3.25
							P=0.003	
AFB ₁ (ng/kg DM)	15.28±11.81 ^a (n=12)	22.84±25.12 ^a (n=12)	56.69±38.48 ^b (n=12)	81.56±35.14 ^c (n=12)	1.25	101.25	19.06 ±19.58	69.13 ±38.21
							P<0.001	
Daily AFB ₁ intake (ng)	496.79±438.57 ^a (n=10)	577.31±680.62 ^a (n=10)	1013.50±820.72 ^a (n=10)	1720.06±889.36 ^b (n=10)	111.63	2551.20	537.05 ±558.79	1375.50 ±905.02
							P=0.002	
Frequencies (n=48) **								
<1.25 ngkg ⁻¹	1	4	1	-	Total		5	1
>101.25ngkg ⁻¹	-	-	-	7			-	7
* Assuming that the values of 1.25 and 101.25ngkg ⁻¹ (the lower and the upper limits of the experimental kit) were the lowest and the highest contamination rates; TMR: total mixed ration								
** Excluding the two farms with incomplete data (totally 40 bunk TMR samples), 5 samples had non-detectable values (4 in winter) and 5 samples had levels above 101.25ng/kg (all in summer)								

Correlations

No correlation was detected in neither of seasons between the ration AFB₁ and milk AFM₁. There were some correlations between the AFB₁ content of TMRs and the amount of AFB₁ added to the ration by each ingredient (Table 5). The most frequent and the strongest correlations were seen for grain mix succeeded

by corn silage, wet beet pulp and dry beet pulp, respectively. The AFB₁ in alfalfa hay was correlated only with that of calculated TMR in the sum of both seasons, irrespective of its higher contaminations compared with the other ingredients. The correlations were less prominent for bunk TMRs.

The AFB₁ levels in calculated and bunk TMRs were related in summer ($r=0.48$, $P=0.033$) and in the sum of both seasons ($r=0.43$, $P=0.006$), but not in winter ($r=-0.05$, $P=0.83$).

Table 5						
Correlations (r values) between the aflatoxin level in calculated or bunk TMR and the aflatoxin added to the ration by each ingredient (P values in parentheses)						
	Calculated TMR			Bunk TMR		
	Winter	Summer	Both seasons	Winter	Summer	Both seasons
Alfalfa hay	-	-	0.31 (0.056)	-	-	-
Corn silage	0.53 (0.016)	0.52 (0.018)	0.66 (0.001)	-	-	0.31 (0.050)
Beet pulp-dry	0.61 (0.036)	-	0.42 (0.053)	-	0.67 (0.033)	-
Beet pulp-wet	-	-	0.68 (0.005)	-	-	-
Grain mix	0.82 (0.001)	0.91 (0.001)	0.90 (0.001)	-	0.45 (0.047)	0.38 (0.015)

Discussion

Aflatoxin M1 in milk

While all bulk milk samples ($n=72$; 100%) showed contaminations with AFM₁, 55 samples (76%) had levels below 81 ngkg⁻¹. The remaining 17 samples (24%) with higher contaminations could potentially be below or above the standard limit of 100 ngkg⁻¹. Twenty-one samples (29%) were contaminated below 50 ngkg⁻¹. The real contaminations could probably be lower than the detected values due to the method of prediction (ELISA). Less AFM₁ values are usually obtained with HPLC method compared with ELISA (Alvarado et al., 2017). A meta-analysis on Iranian studies (Khaneghahi-Abyaneh, et al., 2019) showed means of 59.19 and 35.23 ngkg⁻¹ with ELISA (55 studies; 9224 samples) and HPLC (18 studies; 2606 samples) methods, respectively. Some Iranian studies have reported AFM₁ contamination in UHT and pasteurized milk. Mashak et al. (2016) detected AFM₁ (15 to 140 ngkg⁻¹; HPLC method) in all (100%; $n=30$) samples, 66.7% ($n=20$) having levels below 50 ngkg⁻¹, 20% ($n=6$) above 100 ngkg⁻¹ and none over 500 ngkg⁻¹. Tajik et al., (2016; ELISA method), found AFM₁ (75.8±9.2 ngkg⁻¹) in 77.7% of samples ($n=280/360$), 36% ($n=130$) showing levels below 50 ngkg⁻¹ and 2.2% ($n=8$) exceeding 500 ngkg⁻¹. Heshmati and Milani (2010; ELISA method) found AFM₁ in 55.2% ($n=116/210$) of samples, with 21.9% ($n=46$) having levels below 50 ngkg⁻¹ and none exceeding 500 ngkg⁻¹. Based on a meta-analysis of 70 Iranian studies (Hamzeh-pour et al., 2020), 64% of raw milk samples were contaminated to AFM₁ (mean 39.7 ngkg⁻¹), 75% of which below 50 ngkg⁻¹ and 9% above 500 ngkg⁻¹. Contaminations above 50 ngkg⁻¹ have been reported from other countries (Škrbić et al., 2013; Tsakiris et al., 2013). Škrbić et al. (2013) reported a mean of 300 ngkg⁻¹ AFM₁ in commercial milk samples from Serbia.

Seasonal differences in milk AFM₁ levels have been reported with higher contaminations in cold seasons (Khaneghahi-Abyaneh, et al., 2019; Mozaffari Nejad et al., 2019; De Roma et al., 2017; Mahmoudi and Norian, 2015; Bahrami et al., 2016; Heshmati and Milani, 2010; Kamkar, 2005). In the present study, although the estimated milk contaminations in winter (61.25±28.91 ngkg⁻¹) and summer (54.20±25.51 ngkg⁻¹) were not statistically different ($P=0.279$), the contaminations above 81 ngkg⁻¹ or the median (70 ngkg⁻¹) were mostly detected in winter and the chance of contaminations above median was 5.33 times in winter. Interestingly, the AFB₁ contamination of feeds, as the main source of contamination, was significantly higher in summer than in winter ($P<0.002$). As the main source of milk contamination, the AFB₁ contaminations of feeds have been reported to be higher in winter (Bahrami et al., 2016; Dimitrieska-Stojković et al., 2015; Mahmoudi & Norian, 2015; Simas et al., 2007). In the present study, however, the AFB₁ contaminations of all rations and feed ingredients were significantly higher in summer than in winter (see below).

Aflatoxin B₁ in Feed ingredients and TMRs

Ninety percent of the examined ingredients (n=207/230) were positive for AFB₁, 10% (n=23/230) had non-detectable values and 13.9% (n=32/230) had values above 101.25 ngkg⁻¹. All ingredients exhibited higher contaminations in summer (P<0.05). The non-detectable AFB₁ values were mostly seen in winter (n=17/23) and the values above 101.25 ngkg⁻¹ were detected mainly in summer (n=27/32). These differences could be due to the synergistic effects of ambient temperature and feed moisture on behavior of mycotoxigenic fungi in summer, which is affected by climatic changes at any stage of production chain (Paterson and Lima, 2010; Magan et al., 2011; Guchi, 2015; Alvarado et al., 2017).

Iranian studies focusing on AFB₁ contamination of dairy feeds are not abundant. Bahrami et al. (2016) examined 160 samples of corn silage, alfalfa hay, straw and barley (n=40 each), detected AFB₁ in 82.5% of samples with levels above 5,000 ngkg⁻¹ (Iranian standard) in 65% of corn silage and 10% of straw samples. Different prevalence rates have been reported for aflatoxin contamination of feeds from various countries such as China (42%; Zheng et al., 2013), Costa Rica (33%; Chavarría et al., 2015), Portugal (22%; Abrunhosa et al., 2016), Tanzania (65%, Mohammed et al., 2016) and Turkey (61.7%, Kocasari et al., 2013; 34.9% Sahin et al., 2013; 26.3%, Sahin et al., 2016). A global study covering 2009 to 2011 (Rodrigues and Naehrer, 2012) and 4,627 feed samples reported aflatoxin contamination in 33% of samples (mean: 21,000 ngkg⁻¹). A survey of world reports on 1,113 feed samples from 44 countries between 2012 and 2015 (Kovalsky et al., 2016) reported that only a few samples from Africa and Europe exhibited levels exceeding 20,000 ngkg⁻¹, which is the maximum permitted level for some non-dairy animals (Kunsagi et al., 2011; ISIRI, 2016). Different prevalence rates among various studies might be due to climatic variations and different detection methods. Ghali et al. (2008, 2009) detected aflatoxins in 76.4% and 62% of sorghum samples with ELISA and HPLC methods, respectively.

Consistent with the feed ingredients, the TMRs also had higher contaminations in summer. The total daily AFB₁ contents of bunk TMRs were 537.05±558.79 ng in winter and 1375.50±905.02 ng in summer. It is implied from some evidences that these levels were low enough to result in milk AFM₁ levels below 50 ngkg⁻¹. About 0.3 to 6.2% of AFB₁ is secreted as AFM₁ to milk (Becker-Algeri et al., 2016; FAO/WHO, 2002) and in cows producing >30 kg milk/day, about 6% of AFB₁ may be transformed to AFM₁ (Britzi et al., 2013). In the present study, with milk production of 30.5kg/cow/day and assuming 6% bio-transformation, the AFM₁ content of milk could range from 1.05-2.7 ngkg⁻¹ (totally 32 to 82.5 ng). However, the estimated AFM₁ levels were 61.25±28.91 ngkg⁻¹ in winter and 54.20±25.51 ngkg⁻¹ in summer. Thus, the true contaminations of the ingredients could be higher than the detected levels. As the AFB₁ content of TMR samples taken from bunks were higher than those calculated from the contaminations of separate ingredients, the contamination could be increased during processing. Common devices such as mills and mixing wagons (Pinotti et al., 2016) and remainders of contaminated feeds in wagons, preparation areas and bunks may increase the level of aflatoxin in the ration. The difference could also be due to the small quantities of samples taken from huge volumes of feeds. The lack of correlation between feed AFB₁ and milk AFM₁ could be due to variations in daily intake of AFB₁.

Seasonal variations in AFB₁ in feeds and AFM₁ in milk

With elevated dietary AFB₁ levels and high chance of TMR contaminations above median (716 ng/kg; OD=5.57; P=0.002) in summer, higher levels of AFM₁ in milk would be expected, but the findings were opposite. The reason could be the affection of liver functions by heat stress in summer (Bernabucci et al., 2010; Gallo et al, 2015; Marchese et al., 2018; Min et al., 2021). Transformation of AFB₁ to AFM₁ happens mainly in the liver by the action of cytochrome P450 enzymes (Marchese et al., 2018; Min et al., 2021), involved in a vast variety of reactions. Compromised liver functions and reduced hepatic enzyme activities have been observed in heat stressed cows (Bernabucci et al., 2010; Fan et al., 2018). Compromised liver activities may be long lasting as it was observed in mid lactation cows (Mohebbi-Fani et al., 2020). Lower dry matter intakes in summer could also have a role in this finding. So, non-dietary factors affecting the metabolism of AFB₁ may fade the effect of higher intakes of toxin in summer. Paradoxically, if the general health of the cows is improved, the AFM₁ contamination of milk would potentially be elevated. Thus, the most logic way to control the aflatoxin contamination in milk is to control the critical contaminants of the rations.

Critical feed contaminants

The AFB₁ content of all ration ingredients showed some correlations with AFB₁ content of TMRs. The strongest correlations were seen for grain mix and corn silage, which had the highest proportions in the rations. However, the impact of all other ingredients should also be taken into account. Beet pulp also could be an important source of contamination (strong correlations) regardless of its low incorporation or absence in rations. Alfalfa hay also could be an important contaminant, despite that its AFB₁ contents were not correlated with those of the total rations. It is incorporated in nearly all rations in considerable amounts in Iranian farms and may be substituted for corn silage in many conditions. The co-occurrence and the synergistic effects of various mycotoxins even at relatively low levels (Kovalsky et al., 2016) should be kept in mind. In addition to controlling the AFB₁ contaminations of feed ingredients, tainting of feedstuffs during processing should also be controlled. Intensifying the controlling measures in summer, with probable higher contaminations of feedstuffs, may reduce the overall milk contamination. Responses to these efforts may be rapid as AFM₁ enters the milk 12-24 hours after consumption of AFB₁ and drops to non-detectable levels about 72 hours of removal of AFB₁ from ration (Pettersson, 1998). Based on the results of this study, a great majority of milk produced in the studied farms could have AFM₁ contaminations below the ISIRI limit (100 ngkg⁻¹). Contaminations below 50 ngkg⁻¹ appear to be achievable and affordable in the studied farms.

Declarations

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Authors' contributions

literature review, sampling, data collection and measurements were done by Ravel Gholampour-Aghdami. Designing and managing the work were performed by Mehdi Mohebbi Fani and Arash Omid who also prepared *the first draft of the manuscript*. *Aria Rasooli and Maryam Ansari-Lari advised on raw data and corrected the text. Statistical analysis was performed by Maryam Ansari-Lari.*

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Figures

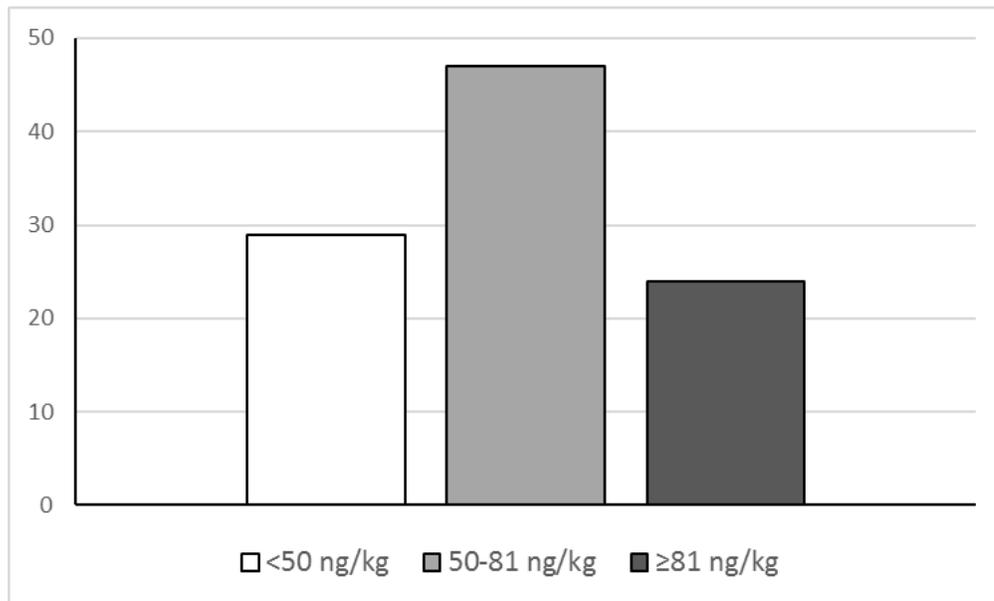


Figure 1

Frequencies (%) of bulk milk samples (n=72) with AFB1 contaminations below 50, 50-81 and above 81 ngkg⁻¹.

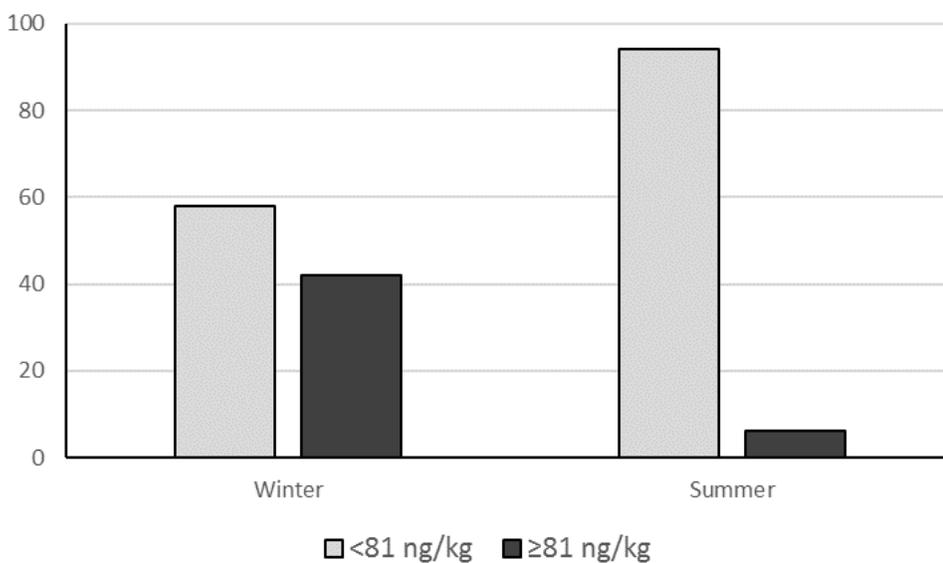


Figure 2

Frequencies (%) of bulk milk samples with AFM1 contaminations below and above 81 ng/kg during winter and summer (n=36 each season).

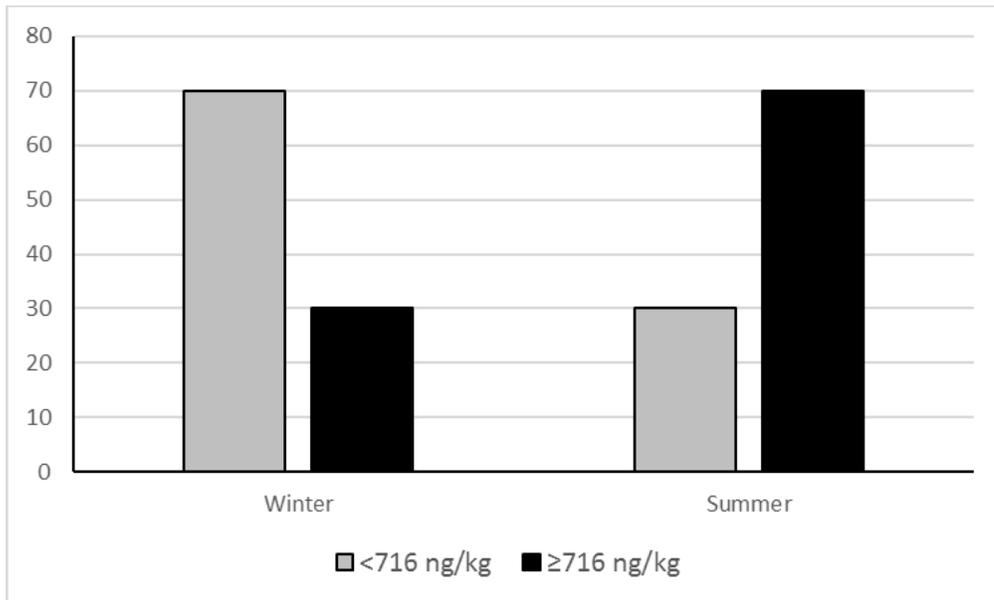


Figure 3

Frequencies (%) of bunk TMR samples with AFB1 contaminations below and above median (716 ng/kg) during winter and summer (n=24 each season).