

A survey on Aflatoxin M₁ content in sheep and goat milk produced in Sardinia Region, Italy (2005-2013)

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Abstract

In the present work the results of a survey conducted in Sardinia Region on Aflatoxin M₁ (AFM₁) contamination in milk of small ruminants from 2005 to 2013 are reported. A total of 517 sheep and 88 goat milk samples from bulk tank, tank trucks and silo tank milk were collected. Analyses were performed by the Regional Farmers Association laboratory using high-performance liquid chromatography following the ISO 14501:1998 standard. None of the sheep milk samples analysed during 2005-2012 showed AFM₁ contamination. In sheep milk samples collected in 2013, 8 out of 172 (4.6%) were contaminated by AFM₁ with a concentration (mean±SD) of 12.59±14.05 ng/L. In one bulk tank milk sample 58.82 ng/L AFM₁ was detected, exceeding the EU limit. In none of goat milk samples analysed from 2010 to 2012 AFM₁ was detected. In 2013, 9 out of 66 goat milk samples (13.6%) showed an AFM₁ concentration of 47.21±19.58 ng/L. Two of these samples exceeded the EU limit, with concentrations of 62.09 and 138.6 ng/L. Higher contamination frequency and concentration rates were detected in bulk tank milk samples collected at farm than in bulk milk truck or silo samples, showing a dilution effect on AFM₁ milk content along small ruminants supply chain. The rate and levels of AFM₁ contamination in sheep and goat milk samples were lower than other countries. However, the small number of milk samples analysed for AFM₁ in Sardinia Region in 2005-2013 give evidence that food business operators check programmes should be improved to ensure an adequate monitoring of AFM₁ contamination in small ruminant dairy chain.

Introduction

An increased frequency of Aflatoxins (AFs) contamination in corn has been reported in the last few years, where feed imported from third countries or crop raised in different European countries, mainly in West and South Europe were implicated (Streit *et al.*, 2012; EU-RASFF, 2014) (Figure 1). Corn and related products are widely used as feedstuff in dairy animals as an important source of fermentable carbohydrates. Recent works also showed an increased AFs contamination rate in corn produced in Italy (Causin, 2013). From 2003 to 2012, regions of Northern Italy – which are the main national corn producers – were affected by particular climatic conditions. An increase in temperature and drought stress caused a high rate of corn crops contaminated with Aflatoxin B₁ (AFB₁). In 2012, during a wide survey conducted on corn produced in Northern Italy, 31.326 samples taken at storage plants were analysed. The results showed that AFB₁ contamination above the EU limit of 20 µg/kg, was detected in samples representative of about 784.000 corn tons, corresponding to 45.2% of the total production (Causin, 2013). A correspondence between AF contamination in corn and the presence of AFs metabolites in Italian cow milk and dairy products was observed (Bolzoni *et al.*, 2013). As a consequence of the last AFs contamination crisis, the Italian Ministry of Health enforced measures to minimise the risk of contamination of milk and dairy products by Aflatoxin M₁ (AFM₁). Preventive measures along bovine dairy production chain and more stringent requirements for food business operators (FBOs) own-check monitoring programmes were established (Italian Ministry of Health, 2013).

Aflatoxin M₁ contamination levels in goat and sheep milk is generally lower as compared to cow's milk (Virdis *et al.*, 2008). Sheep and goat mainly graze on pasture and their lower intake in concentrate reduces the exposure to AFs. The use of concentrate and feedstuff in the formulation of small ruminants feeding is limited due to economic reasons and to effectiveness on milk production (Molle *et al.*, 2008).

The ability to convert the AFB₁ ingested with feedstuff to AFM₁ excreted with milk, referred to as carry-over, is also variable between large and small ruminants. Previous works reported carry-over values ranging between 0.35 and 3% in cows (Veldman *et al.*, 1992; Frobisch *et al.*, 1986) and between 0.018 and 3.1% in goats (Goto and Hsieh, 1985; Nageswara Rao and Chopra, 2001; Ronchi *et al.*, 2005). Lower carry-over rates were found in dairy sheep, ranging between 0.08 and 0.33% (Battacone *et al.*, 2005).

The own-check programmes developed in

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Sardinia Region by FBOs in the last years included monitoring for AFM₁ in sheep and goat milk only in few cases. Only few cheese-making factories conducted AFM₁ analysis by means of rapid detection methods on internal laboratories. Most of the analyses for AFM₁ detection were carried out at the Regional Farmers Association laboratory using high-performance liquid chromatography (HPLC).

In the current work the results of the AFM₁ monitoring programme conducted in Sardinia Region on sheep and goat milk during eight years period, from 2005 to 2013, are presented.

Materials and Methods

During the period from 2005 to 2013 a total of 517 sheep milk and 88 goat milk samples were collected for the detection of AFM₁. Samples were represented by: 75 bulk tank milk samples, of which 56 and 19 were obtained from sheep and goat farms, respectively; 443 milk tank trucks samples, 401 from sheep and 42 from goat farms and 87 milk samples from silo tanks (60 from sheep milk and 27 from goat milk processing plants). All the analysis were performed by the Regional Farmers Association laboratory using the HPLC 1100 series (Agilent Technologies Inc., Santa Clara, CA, USA) with automatic sampler LAS G1313A and a fluorescence detector (FLD) G1321, following the ISO 14501:1998 standard.

After AFM₁ extraction, samples were processed using HPLC-FLD method. Briefly, 50 mL of each milk sample were centrifuged at 4000 r/min for 15 min to separate the fat fraction and the skimmed sample injected into an immune-affinity columns (VICAM) with a flow of 2 mL/min. Each column was washed with 10 mL of ultrapure water (MillQ; Millipore, Billerica, MA, USA) with a flow of 2 mL/min and the AFM₁ eluted from the column using 4 mL of acetonitrile. Then, the eluate was dried at 45-50°C with a nitrogen flow and the dried residue resuspended with 500 µL of water-methanol (50:50 w/v). Finally, 10 µL of the solution were loaded into a Zorbax SB C18 column 150×4.6 mm with a 5 µm diameter (Agilent Technologies Inc.). The mobile phase (water-methanol-acetonitrile, 63:26:11 w/v) was injected with a flow of 1 mL/min in isocratic condition. All the standard for the AFM₁ detection were dissolved in methanol-water solution (10 µg/mL) and stored at 4°C until use. The calibration curve was determined by loading 5 AFM₁ standard solution at the concentration of 0.012, 0.025, 0.050, 0.100, 0.200 and 0.300 µg/L.

(Table 2) bulk tank milk samples collected at farm level, from trucks and from silo tanks were analysed for the detection of AFM₁. In all milk samples collected from 2005 to 2012, 345 (66.7%) and 22 (25%) from sheep and goats, respectively, the presence of AFM₁ was never detected. For both species the presence of AFM₁ was observed only in samples collected in 2013, when were analysed 172 (33.3%) milk samples from sheep and 66 (75%) from goat. Of sheep milk samples collected in 2013, 8

(4.6%) showed an AFM₁ contamination greater than 8 ng/L, with a concentration (mean±SD) of 12.59±14.05 ng/L, range between 8.72 and 58.82 ng/L. In two bulk tank milk samples (7.4%) collected from sheep farms AFM₁ was detected (34.19±34.83 ng/L), and in one of these a concentration of 58.82 ng/L, exceeding the EU limit. Aflatoxin M₁ was also detected in 4 samples (4.5%) from tank trucks (13.54±6.80 ng/L, range between 9.48 and 23.71 ng/L) and in 2 samples (3.6%) from silo (13.67±6.99

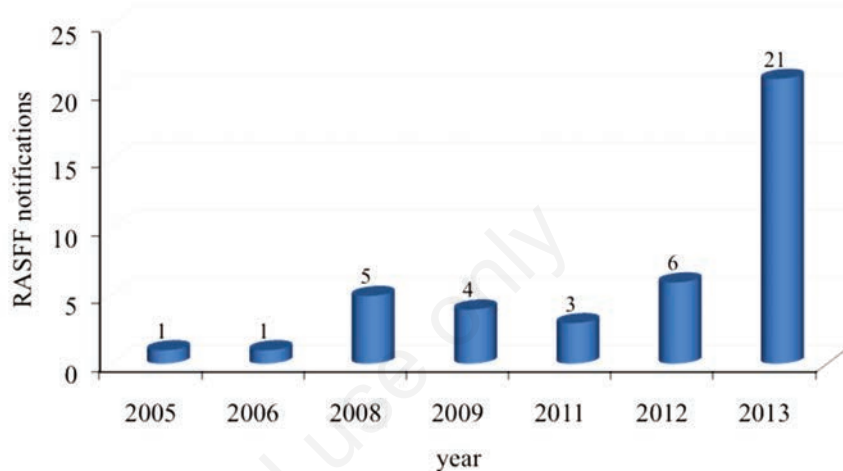


Figure 1. Rapid Alert System for Food and Feed notification on Aflatoxins contamination in corn used for feedstuffs production from 2005 to 2013.

Results

A total of 517 sheep (Table 1) and 88 goat

Table 1. Concentration of Aflatoxin M₁ detected in sheep milk samples collected from 2005 to 2013 using the high-performance liquid chromatography-fluorescence detector method.

Year	Samples (n)	AFM ₁ concentration (ng/L)			
		<8	≥8-20	>20-50	>50
2005	12	12	-	-	-
2006	58	58	-	-	-
2007	51	51	-	-	-
2008	46	46	-	-	-
2009	52	52	-	-	-
2010	40	40	-	-	-
2011	41	41	-	-	-
2012	45	45	-	-	-
2013	172	164	6	1	1
Total	517	509	6	1	1

AFM₁, Aflatoxin M₁.

Table 2. Concentration of Aflatoxin M₁ detected in goat milk samples collected from 2010 to 2013 using the high-performance liquid chromatography-fluorescence detector method.

Year	Samples (n)	AFM ₁ concentration (ng/L)			
		<8	≥8-20	>20-50	>50
2010	4	4	-	-	-
2011	5	5	-	-	-
2012	13	13	-	-	-
2013	66	57	2	5	2
Total	88	79	2	5	2

AFM₁, Aflatoxin M₁.

ng/L). A summary of AFM₁ contamination levels in sheep milk samples collected in 2013 is reported in Table 3.

In 9 (13.6%) out of 66 goat milk samples collected in 2013, AFM₁ was detected at a concentration of 47.21±19.58 ng/L (Table 4), range between 10.45 and 138.16 ng/L. Contamination by AFM₁ was observed also in 2 samples (22.2%) from bulk tank milk (80.00±82.25 ng/L), in 6 samples from milk tank trucks (34.38±19.92 ng/L, range between 10.45 and 62.09 ng/L) and in 1 sample (3.8%) from silo tank (30.40 ng/L). AFM₁ was detected at a con-

centration exceeding the EU limit in one bulk tank milk sample (138.6 ng/L) and in 1 tank truck milk sample (62.09 ng/L).

Discussion

In the past years, the monitoring of AFM₁ contamination in milk of small ruminants in Sardinia Region has been carried out only on a small number of samples. However, in 2013 AFM₁ contamination in cow milk and in several

cases also in sheep and goat milk was reported. With respect to the crisis occurred in Sardinia Region in 2003, the last one was better managed with a faster response of the public veterinary authorities. As in other areas of Italy this was mainly due to the experience gained in previous emergencies (Bolzoni *et al.*, 2013). Therefore, from 2013 the Competent Authority increased the official control on AFs contamination throughout the small ruminants dairy chain (Sardinia Region, 2013). In the same year, the number of milk samples analysed in the own-check monitoring programme showed

Table 3. Detection of Aflatoxin M₁ in sheep milk samples collected from bulk tank, milk tank truck and silo tank using the high-performance liquid chromatography-fluorescence detector method.

Tank	Milk samples (n)	AFM ₁ concentration (ng/L)			
		<8	≥8-20	>20-50	>50
Bulk	27	25 (92.6)	1 (3.7)	-	1 (3.7)
Milk truck	89	85 (95.5)	3 (3.4)	1 (1.1)	-
Silo	56	54 (96.4)	2 (3.6)	-	-
Total	172	164 (95.3)	6 (3.5)	1 (0.6)	1 (0.6)

AFM₁, Aflatoxin M₁.

Table 4. Detection of Aflatoxin M₁ in goat milk samples collected from bulk tank, milk tank truck and silo tank using the high-performance liquid chromatography-fluorescence detector method.

Tank	Milk samples (n)	AFM ₁ concentration (ng/L)			
		<8	≥8-20	>20-50	>50
Bulk	9	7 (77.8)	-	1 (11.1)	1 (11.1)
Milk truck	31	25 (80.6)	2 (6.5)	3 (9.7)	1 (3.2)
Silo	26	25 (96.2)	-	1 (3.8)	-
Total	66	57 (86.4)	2 (3.0)	5 (7.6)	2 (3.0)

AFM₁, Aflatoxin M₁. Values in brackets are expressed as percentage.

Table 5. Aflatoxin M₁ occurrence in bulk tank sheep milk samples in different countries.

Year	Samples (n)	Country	Positive samples (%)	Mean±SD (ng/L)	Detection method	References
2005-2006	23	Syria	13 (57)	67±18.4	ELISA	Ghanem and Orfi, 2009
2007	24	Pakistan	4 (16.7)	2.0±4.0°	HPLC	Hussain <i>et al.</i> , 2010
2007-2008	51	Iran	19 (37.3)	28.1±13.7	ELISA	Rahimi <i>et al.</i> , 2010
2007-2008	814	Spain	387 (47.5)	-	ELISA	Rubio <i>et al.</i> , 2011
2008-2009	42	Iran	13 (31.0)	25.8±15.1	ELISA	Rahimi and Ameri, 2012
2009	118	Italy	1 (0.8)	5.2	ELISA	Cossu <i>et al.</i> , 2011
2013	19	Croatia	0 (0.0)	3.7±0.91°	ELISA	Bilandžić <i>et al.</i> , 2014

SD, standard deviation; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography. °Concentration mean was determined on all the samples.

Table 6. Aflatoxin M₁ occurrence in bulk tank raw goat milk samples in different countries.

Year	Samples (n)	Country	Positive samples (%)	Mean±SD (ng/L)	Detection method	References
2003-2004	208	Italy	36 (17.3)	14.5±8.4	ELISA	Viridis <i>et al.</i> , 2008
2005-2006	11	Syria	7 (64)	19±13.8	ELISA	Ghanem and Orfi, 2009
2007	30	Pakistan	6 (20.0)	2.0±5.0°	HPLC	Hussain <i>et al.</i> , 2010
2007-2008	60	Iran	19 (31.7)	30.1±18.3	ELISA	Rahimi <i>et al.</i> , 2010
2008-2009	48	Iran	17 (35.4)	31.8±13.7	ELISA	Rahimi and Ameri, 2012
2013	32	Croatia	2 (6.2)	7.6±8.94°	ELISA	Bilandžić <i>et al.</i> , 2014

SD, standard deviation; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography. °Concentration mean was determined on all the samples.

an increasing trend (Tables 1 and 2). However, the number of small ruminants milk samples investigated for AFM₁ detection is still limited and it should be increased (Tables 5 and 6). Until 2012, AFM₁ contamination in all analysed samples was not detectable. On the other hand, in 2013, several sheep (7.4%) and goat (22.2%) milk samples were found contaminated with AFM₁. Previous works conducted in the same production areas using the ELISA detection method, showed a prevalence of positive samples of 0.8 and 17.3% for sheep and goat milk, respectively (Virdis *et al.*, 2008; Cossu *et al.*, 2011). However, the present study demonstrated that in Sardinia Region, the prevalence of sheep milk samples contaminated with AFM₁ was lower than reported in other countries (Table 5).

Conclusions

The current own-check monitoring programme on AFM₁ contamination in milk of small ruminants produced in Sardinia Region require a reevaluation of framework arrangement and resources. A larger amount of samples should be analysed to cover a large number of milk producers and cheese-making factories. The wider use of rapid screening test should be promoted, limiting HPLC as a confirmatory method. Developing own-check monitoring and controlling programmes in small ruminant dairy chains is more complex as compared to the dairy cow sector. This is mainly related to the high number of sheep and goat farms that should be submitted to a monitoring plan, while a limited amount of milk per farm is delivered to the cheese making plants. For this reason, in order to evaluate the AFM₁ contamination levels, the FBOs have increased the number of tank truck and silo tank milk samples (Tables 3 and 4). In small ruminant dairy chains, tank truck and silo gather bulk milk from a larger number of farms than in cows, resulting in potential stronger dilution effect of contaminants in the milk delivered. In the present work a reduction of AFM₁ concentration and rate of positive samples was observed in relation to the origin of samples, decreasing from samples taken at farm level to truck tank and silo. In Italy, an attention level of 40 ng/kg for AFM₁ in bulk tank cow milk was established. In small ruminants dairy chains, the own-check pro-

grammes for monitoring AFM₁ consider the milk tank truck as a main target and so attention level should be set at a lower level than those provided for cows, due to the observed dilution effect of the contamination.

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