Transfer of Aflatoxin B1 from Feed to Milk and from Milk to Curd and Whey in Dairy Sheep Fed Artificially Contaminated Concentrates

G. Battacone,¹ A. Nudda,¹ M. Palomba,² M. Pascale,³ P. Nicolussi,⁴ and G. Pulina¹

¹Dipartimento di Scienze Zootecniche, Università degli Studi di Sassari, Via De Nicola 9, 07100 Sassari, Italy

²Dipartimento Farmaco Chimico Tossicologico, Università degli Studi di Sassari, Via Muroni 23/A, 07100 Sassari, Italy

³Istituto di Scienze delle Produzioni Alimentari CNR, Via Amendola, 122/o 70126 Bari, Italy

⁴Istituto Zooprofilattico Sperimentale della Sardegna, Via Duca degli Abruzzi 8, 07100 Sassari, Italy

ABSTRACT

An experiment was carried out using dairy ewes to study the transfer of aflatoxin B1 (AFB1) from feed to milk and from milk to cheese. The effects of AFB1 on liver function and hematological parameters were also investigated. Fifteen ewes were assigned to treatments in replicated 3 × 3 Latin squares. The experimental groups received 32, 64, or 128 µg/d of pure AFB1 for 7 d followed by 5 d of clearance. On the sixth day of the first period, the total daily milk produced by each ewe was collected separately and processed into cheese. The results indicate that the level of AFB1 used did not adversely affect animal health and milk production traits. The aflatoxin M1 (AFM1) concentrations in milk approached a steady-state condition in all treated groups between 2 and 7 d after the start of treatment. The mean AFM1 concentrations of treated groups in steady-state condition (184.4, 324.7, and 596.9 ng/kg in ewes fed 32, 64, or 128 µg of AFB1, respectively) were significantly affected by the AFB1 doses. The AFM1 concentration was linearly related to the AFB1 intake/kg of BW. The carry-over values of AFB1 from feed into AFM1 in milk (0.26 to 0.33%) were not influenced by the AFB1 doses. The AFM1 concentrations in curd and whey were linearly related to the AFM1 concentrations in the unprocessed milk.

(Key words: aflatoxin, dairy sheep, carry-over, milk)

Abbreviation key: AFB1 = aflatoxin B1, AFM1 = aflatoxin M1.

INTRODUCTION

Aflatoxins are a group of fungal toxins, produced mainly by *Aspergillus flavus* and *A. parasiticus*, which

occur naturally in several important feedstuffs. Major crops in which aflatoxins are produced are peanuts, corn, and cottonseed. Aflatoxin B1 (AFB1) is considered to be the most toxic compound produced by these molds. In the liver, ingested AFB1 is biotransformed by hepatic microsomal cytochrome P450 into aflatoxin M1 (AFM1), which is then excreted into the milk of lactating animals.

The International Agency for Research on Cancer of WHO (IARC, 2002) includes aflatoxins among the substances that are carcinogenic for humans (Group 1). Several countries have regulated the maximum permissible levels of AFB1 in food and AFM1 in milk and dairy products. The European Community has established that maximum levels of AFM1 in liquid milk and dried or processed milk products should not exceed 50 ng/kg and that when the maximum level is being established for processed milk, one must take into consideration changes in the concentration of the contaminant caused by processing (European Commission, 2001). By contrast, the maximum AFM1 concentration level established by the FDA is 500 ng/kg in milk, and the FAO/WHO Joint Expert Committee on Food Additives have accepted this limit (Berg, 2003).

One of the principal ways by which aflatoxins are introduced into the human diet is through consumption of milk and milk products (Galvano et al., 1998).

Roussi et al. (2002) reported that the incidence of AFM1 contamination in raw milk samples from sheep (obtained from different milk producers across Greece) were 66.7% in 2000 and 73.3% in 2001, with only one raw sheep milk sample exceeding the EU limit.

When pure AFB1 was given to ewes fed high NDF diets, the AFM1 concentrations in the milk increased within 12 to 144 h of the beginning of administration. Milk AFM1 concentration measured in steady-state conditions was significantly affected by the AFB1 dose (Battacone et al., 2003). The carry-over ratio (AFM1 excreted in milk/AFB1 ingested) has been found to be

Received March 1, 2005.

Accepted June 7, 2005.

Corresponding author: G. Pulina; e-mail: gpulina@uniss.it.

lower in sheep (Battacone et al., 2003) than in cattle (Veldman et al., 1992). As AFM1 is linked to milk proteins, its concentration is higher in curd than it is in milk. The AFM1 concentration in milk was not influenced by the milk production level in cattle. This means that the total amount of AFM1 excreted in milk and, consequently, the carry-over ratio, increased with milk yield (Munksgaard et al., 1987; Veldman et al., 1992). A previous study carried out on iso-productive dairy ewes found that as the amount of AFB1 ingested increased, the AFM1 concentration in both milk and curd also rose (Battacone et al., 2002). An experimental trial was carried out using dairy ewes to check the transfer of AFB1 from feed to milk and from milk to cheese. The effects of AFB1 on liver function and hematological parameters were also investigated.

MATERIALS AND METHODS

Experimental Procedures

Twenty Sarda ewes in late lactation (>120 DIM) and with a BW of 48 ± 6 kg (mean $\pm$ SD) were used. The ewes were milked twice daily at 0700 and 1900 h and fed a concentrate mixture (CP = 17.8%, NDF = 33.7%; DM basis) of 750 g/d per head plus grass hay (CP = 5.2%, NDF = 66.5%; DM basis) for ad libitum intake. Fifteen ewes were allocated to 5 simultaneous balanced 3×3 Latin squares and received 3 treatments. Five additional ewes, the control group, received an aflatoxin-free diet. The treatments were 32 μ g (T1), 64 μ g (T2), and 128 μ g (T3) of AFB1/d per head, divided in two equal daily doses. The treated animals were fed a pellet artificially contaminated with AFB1 immediately before each milking. Pure AFB1 was dissolved in methanol, and a pellet of concentrate was used as the aflatoxin carrier (Sigma A-6636; Sigma Chemical Co., St. Louis, MO). The treatment lasted 7 d with a 5-d interval between periods (clearance). The milk yield of each ewe was recorded and sampled at each milking throughout the experiment and the subsequent clearance periods. Milk samples were stored at -18°C until the AFM1 content could be analyzed. The milk samples from the seventh day of administration were also analyzed for fat, protein ($N \times 6.38$), and SCC.

Blood samples were collected by jugular veinpuncture on the seventh day of administration and immediately analyzed to check effects of the treatments on liver function and hematological parameters.

On the sixth day of the first period, the total daily milk produced by each ewe was collected separately and processed into cheese. Raw milk (750 mL) was heated at 36 to 37°C , and 0.15 mL of standardized rennet solution (1:15000; Chr. Hansen, Corsico, Milano, Italy) was added. The milk was left undisturbed for about 30 min

to allow for coagulation. The curd was then cut and stirred gently at 35 to 36°C for 30 min. The curd was pressed, and whey was drained at room temperature for 24 h.

The experiments on the animals followed the guidelines of the Council Directive of EC (European Communities, 1986). Before and during the experimental period, the health of animals was monitored continuously.

Analytical Procedures

An immunoaffinity technique was used to extract the AFM1 from the milk. The concentration was determined by HPLC. Fifty milliliters of milk was defatted by centrifuging at 3500 rpm, filtered with filter paper (Whatman no. 4), and then passed through an immunoaffinity column (Afla M1; Vicam LP, Watertown). The column was washed with de-ionized water and then dried under nitrogen. After drying, the AFM1 was eluted with methanol and evaporated to dryness under nitrogen at 45°C . The resultant residue was dissolved in 250 μ L acetonitrile/water (25:75 vol/vol) + 1% acetic acid.

The AFM1 was separated on a Hewlett Packard 1100 HPLC chromatograph connected to a reverse-phase C18 column (Zorbax SB, 5- μ m particle size, 150×4.6 mm i.d.) equipped with a Hewlett Packard 1100 fluorescence detector with excitation at 365 nm and emission at 435 nm. The eluent was acetonitrile/water (25:75 vol/vol) + 1% acetic acid using a flow rate of 1 mL/min. Standard AFM1 (Sigma A-6428; Sigma-Chemical Co.) was dissolved in benzene-acetonitrile at a ratio of 9:1 to prepare a series of working solutions containing 0.001 to 0.5 ng/ μ L. The calibration curve was prepared by plotting the peak area for each standard against the quantity of AFM1 injected. The equation of the calibration curve was used to compute the AFM1 content of the samples.

The carry over of AFM1 in milk was calculated as the ratio between the AFM1 excreted in milk and the intake of AFB1 at the time when the toxin output in milk reached a steady state.

Milk was analyzed for fat and protein with a Milko-scan 6000 (Foss Electric, Denmark) and for SCC with a Fossomatic 360 (Foss Electric).

A clinical chemistry system (Dimension RXL, Dade Behring) was used to analyze the blood samples for total bilirubin, creatinine, glutamic oxlactic transaminase, glutamic pyruvic transaminase, gamma glutamyl transpeptidase, alkaline phosphatase, and lactate dehydrogenase. The red blood cell count, white blood cell count, mean corpuscular volume, hemoglobin, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelets were measured using an

electronic particle counter (MS9; Melet Schloesing Laboratoires, France).

Milk, curd, and whey were collected and analyzed for chemical composition and AFM1 concentration. The methods described previously were used to determine the concentrations of AFM1 in the milk and whey. The AFM1 in curd was extracted using the method reported by Pietri et al. (1997), with some modifications. A sample of grated curd (20 g) was extracted with chloroform (75 mL), celite 545 (5 g), and 1 mL of saturated sodium chloride. The mixture was shaken for 45 min and then filtered through paper. The chloroform extract was evaporated to dryness under vacuum, and the residue was recovered with hexane, methanol, and water (50:1:30 vol/vol/vol). After gentle swirling, the mixture was transferred to a separating funnel and washed (2 × 10 mL of water) and shaken. The lower layer was collected, and AFM1 was extracted with an immunoaffinity column in the form of skimmed milk.

Milk and whey samples were analyzed for fat using Gerber's method and also for protein ($N \times 6.38$) (IDF, 1986) and for casein using IDF methods (IDF, 1964).

The curd moisture content was determined by drying a 3-g sample in a forced-air oven at 100°C for 24 h (AOAC, 2000). The fat content was determined using the Gerber-Van Gulik method (ISO, 1975). Crude protein ($N \times 6.38$) was determined using the IDF Kjeldahl procedure (IDF, 1986).

Statistical Procedures

Milk. The Latin square was analyzed to check for residual effects of treatment using the method reported by Morris (1999). As the residual effect was never significant, data were submitted to GLM-ANOVA. Treatment and period were fixed effects, and block was a random effect. The SCC were divided by 1000 and converted to the natural logarithm before statistical analysis.

Multiple regression analysis was carried out to estimate the milk AFM1 concentration as a function of the amount of AFB1 intake/kg of BW and the milk yield.

Cheese making. Data were submitted to one-way ANOVA to test differences between treatments, and linear regression analysis was carried out to test AFM1 concentrations in curd and whey in comparison with those of milk.

Blood variables. Given the direct or transformed non-normal distribution of most of the blood parameters (Dimauro et al., 2005), differences among treatments were tested with a Kruskal-Wallis nonparametric analysis, and data are presented as median (percentile 25th-percentile 75th).

RESULTS AND DISCUSSION

AFM1 Excretion in Milk

Before the administration of AFB1, the milk samples of all animals did not contain AFM1. No AFM1 was found in the milk from the control group during the experiment. The pattern of AFM1 concentration in the milk of groups T1, T2, and T3 is reported in Figure 1. The AFM1 was detected in milk of all treated ewes within 12 h of the beginning of administration. Mean AFM1 concentrations in milk approached a steady-state condition (or plateau) in all treated groups between 2 and 7 d after the start of treatment. On d 7, AFB1 administration was stopped, and the mean AFM1 concentrations decreased quickly. After 4 d, AFM1 was no longer detected in any of the treated groups. The pattern of AFM1 concentrations clearly demonstrates that the time at which AFM1 is no longer detectable in milk is not related to the AFB1 dose, as already observed in previous studies on sheep (Battaccone et al., 2003) and cows (Frobish et al., 1986). In our previous study, the concentrations of AFM1 in the milk of sheep fed the same doses of pure AFB1 (Battaccone et al., 2003) increased from 0 to 144 h and reached a steady-state condition after 216 h. The different kinetics of AFM1 in milk could be explained by the different rations used in the experiments. The diet of the previous experiment contained more fiber and, thus, allowed lower ruminal transit of digesta and, consequently, greater decontamination activity by the ruminal microorganisms (Westlake et al., 1989).

Mean AFM1 concentrations of treated groups in steady-state conditions (2 to 7 d) are reported in Table 1. The AFM1 concentration increased linearly with the AFB1 dose administered. The AFM1 values were 3- to 6-fold higher than those observed in our previous experiment using the same doses of AFB1 (Battaccone et al., 2003). The difference in the diets in the 2 experiments might have influenced the amount of rumen AFB1 degradation and, as a result, the amount of AFM1 in the milk. In the milk of the T1 group, the AFM1 content was >3 times higher than the EC maximum tolerance level (50 ng/kg), although only in the T3 group did it exceed the FDA maximum tolerance level (500 ng/kg).

The carry over of AFB1 from feed into AFM1 in milk was not influenced by the AFB1 doses. Our results differ from those of previous studies on sheep (Battaccone et al., 2003) and cows (Frobish et al., 1986; Veldman et al., 1992). This result is due to the proportional increase of AFM1 concentrations in milk as AFB1 ingestion increases. The carry-over values (0.26 to 0.33%) are higher than those observed in an earlier study on sheep (0.08 to 0.13%; Battaccone et al., 2003) but are similar

Figure 1. Aflatoxin M1 (AFM1) concentrations (mean $\pm$ SE) in the milk of ewes fed 32 (T1), 64 (T2), and 128 (T3) μ g of AFB1/d for 7 d. For each treatment, each point represents the mean of 15 data.

to the data reported (0.40%) for goats receiving doses of AFB1 (124.4 μ g/d) similar to those used in the T3 treatment (Nageswara Rao and Chopra, 2001).

Because the multiple regression analysis showed no significant estimated values for intercept ($P = 0.48$) and milk yield ($P = 0.77$), the relationship between milk AFM1 concentration and AFB1 intake/kg of BW was expressed using the equation:

$$\text{AFM1 (ng/kg)} = 209 \text{ AFB1 } (\mu\text{g/kg});$$

$$\text{SE} = 103; R^2(\text{corr.}) = 0.68; P < 0.001.$$

Assuming the administration of 1 kg of concentrate for dairy sheep (average BW = 45 kg) contaminated by AFB1 at the EU tolerance level (European Commission, 2003) for lactating animals (0.005 mg/kg), the expected

mean AFM1 concentration in milk (23 ng/kg) is below the EU tolerance level. However, if the shepherds feed sheep with a commonly used concentrate that contains AFB1 at near the EU tolerance level (0.02 mg/kg), the AFM1 concentration in the milk rises above the EU threshold. Similar results were reported for dairy cattle fed naturally contaminated feeds by Veldman et al. (1992) and by Munksgaard et al. (1987). It is worth noting that, for both sheep and cows in the cases just reported, the AFM1 levels in milk are well below the USDA limits.

The milk production traits of the treated animals are summarized in Table 2. The average daily milk yield did not differ in sheep given the different treatments. The milk production of dairy cows was totally unaffected by AFB1 consumption (Frobish et al., 1986; Polan et al., 1974), whereas our previous data (Battacone et

Table 1. Means of concentration of aflatoxin M1 (AFM1) and carry over in milk of ewes receiving different daily doses of aflatoxin B1 (AFB1) (T1 = 32, T2 = 64, and T3 = 128 μ g/d).

	Treatment			SEM	P
	T1	T2	T3		
AFM1, ng/kg	184.4 ^a	324.7 ^b	596.9 ^c	26.15	0.000
Carry over, ¹ %	0.33 ^c	0.29 ^{cd}	0.26 ^d	0.022	0.109

^{a,b}Means within a row with different superscripts differ ($P < 0.001$).

^{c,d}Means within a row with different superscripts differ ($P < 0.11$).

¹Carry over = percentage of AFB1 that was converted to AFM1 and excreted in milk.

Table 2. Means of milk production traits of ewes receiving different daily doses of aflatoxin B1 (T1 = 32; T2 = 64, and T3 = 128 $\mu\text{g/d}$).

	Treatment			SEM	P
	T1	T2	T3		
Milk, kg/d	1.12	1.15	1.03	0.095	0.718
Fat, %	5.98	6.25	6.01	0.195	0.597
Protein, %	4.87	4.79	4.96	0.141	0.717
Ln SCC, $\times 10^3/\text{mL}$	4.42	5.02	5.11	0.381	0.435

al., 2003) showed an unexplained increase in milk yield as the quantity of AFB1 administered increased. Fat, protein concentration, and SCC (Table 2) were not influenced by consumption of AFB1.

The intake of AFB1 did not cause statistically significant changes in all hematological parameters (Table 3). The values were within the physiological range throughout the experimental period. In a previous experiment, where the period of intoxication was longer (2 wk), we found significantly high GPT activity in sheep fed 128 $\mu\text{g/d}$ of AFB1 (Battaccone et al., 2003). A transient alteration of enzymatic activities in the livers of lambs fed higher AF doses (3.85 to 5.42 mg/d) for a longer period was observed by Edrington et al. (1994).

Transfer of AFM1 from Milk to Curd and Whey

The composition of curd and whey, similar to that of milk, was not influenced by AFB1 intake (Table 4). The AFM1 concentration in curd was about twice that of the respective milk for all treatments. This figure is

Table 4. Aflatoxin M1 (AFM1) concentration and composition of milk, curd, and whey. Milk was sampled at sixth day of AFB1 administration from ewes fed different daily doses of AFB1 (T1 = 32, T2 = 64, and T3 = 128 $\mu\text{g/d}$).

	Treatment			SEM	<i>P</i>
	T1	T2	T3		
Milk					
AFM1, ng/kg	151.1	265.7	512.2	37.86	0.000
Fat, %	6.3	6.0	5.6	0.26	0.263
Protein, %	4.4	4.6	4.4	0.19	0.711
Casein, %	3.4	3.5	3.5	0.10	0.874
Curd					
AFM1, ng/kg	369.3	545.2	1028.3	121.84	0.007
Fat, %	25.2	26.5	25.9	0.68	0.424
Protein, %	20.5	20.5	21.3	0.74	0.693
DM, %	49.5	50.2	51.4	0.83	0.314
Whey					
AFM1, ng/kg	112.1	185.5	365.7	20.34	0.000
Fat, %	1.9	1.7	1.3	0.17	0.115
Protein, %	1.2	1.2	1.2	0.09	0.876

near that observed when making cheese from naturally contaminated sheep milk (1.8 to 2.9; Battaccone et al., 2002) and cow milk (2.4 to 2.8; Kiermeier and Buchner, 1977). The regression analyses between AFM1 concentration in milk and curd and milk and whey (Figure 2) show that the AFM1 concentration in milk is a good predictor of AFM1 concentration in curd and whey. The recovery of AFM1 during cheese making was 95.8%. This was distributed 36.9% in curd and 58.9% in whey. These values are close to the data reported by Kiermeier and Buchner (1977) for making cheese from naturally contaminated milk (93.7 to 98.9%) and by Lopez et al. (2001) for artificially contaminated milk (97.3%).

Table 3. Medians [25th–75th percentile] of serum and hematological parameters¹ in ewes that received different daily doses of aflatoxin B1 (AFB1) (T1 = 32, T2 = 64, and T3 = 128 $\mu\text{g/d}$).

	AFB1			P
	T1	T2	T3	
Creatinine, mg dL	0.85 [0.78–0.98]	0.87 [0.77–0.94]	0.84 [0.77–0.90]	0.831
Bilirubin, mg/dL	0.15 [0.12–0.25]	0.18 [0.12–0.30]	0.18 [0.10–0.25]	0.862
GOT, U/L	123 [109–144]	124 [117–135]	128 [112–147]	0.890
GPT, U/L	14 [11–15]	14 [11–15]	14 [11–15]	0.897
GGT, U/L	78 [72–83]	77 [73–79]	76 [72–84]	0.981
ALP, U/L	126 [86–238]	129 [86–247]	119 [99–237]	0.979
LDH, U/L	1190 [1028–1339]	1258 [1154–1329]	1149 [1019–1308]	0.585
Ca, mg/dL	10.4 [9.7–11.1]	10.2 [9.8–11.0]	10.1 [9.9–10.9]	0.801
P, mg/dL	5.2 [4.2–5.8]	5.1 [4.4–5.7]	5.0 [4.3–5.6]	0.799
RBC, $\times 10^{12}/\text{L}$	8.3 [7.6–8.7]	8.2 [7.5–8.8]	8.3 [7.7–8.5]	0.888
WBC, $\times 10^9/\text{L}$	4.1 [3.8–4.8]	4.0 [3.8–4.3]	4.2 [3.3–4.8]	0.734
MCV, fL	47.5 [45.1–49.8]	47.9 [44.7–49.4]	48.2 [44.8–49.7]	0.956
HB, %	8.8 [8.1–9.1]	8.2 [7.9–8.8]	8.6 [8.3–9.0]	0.439
MCH, pg	10.4 [9.7–11.1]	10.0 [9.6–10.6]	10.5 [9.6–11.5]	0.747
MCHC, g/dL	22.2 [20.8–22.9]	21.8 [21.0–23.6]	22.2 [21.7–22.8]	0.974
PLT, $\times 10^9/\text{L}$	166 [147–233]	180 [164–206]	181 [164–223]	0.957

¹GOT = glutamic oxalacetic transaminase, GPT = glutamic pyruvic transaminase, GGT = gamma glutamyl transpeptidase, ALP = alkaline phosphatase, LDH = lactate dehydrogenase, RBC = red blood cell count, WBC = white blood cell count, MCV = mean corpuscular volume (femtoliters), HB = hemoglobin, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, and PLT = platelets.

Figure 2. Relationship of aflatoxin M1 (AFM1) concentration in curd and in whey with milk from which they were made.

CONCLUSIONS

The continuous administration of AFB1 for 7 d to lactating dairy ewes showed that the AFM1 concentration in the milk rose as increased amounts of AFB1 were ingested. The carry over was not affected by the doses of AFB1 and the milk production levels. The AFM1 concentration in curd is strongly dependent on the AFM1 concentrations in the unprocessed milk and was some 2-fold higher than it was in milk.

The lack of residual effects detected suggests that the Latin square is an appropriate experimental design for mycotoxicological trials.

Practical Implications

In the dairy sheep industry, the equations of AFB1 carry over from feed to milk and of AFM1 transfer from milk to curd and whey can be conveniently used by feed-processors and shepherds to avoid contamination in milk exceeding the legally permitted limits and by cheese-makers to estimate the expected AFM1 concentration in cheese made from contaminated milk.

ACKNOWLEDGMENTS

The authors thank Alessandro Mazzette for his invaluable technical assistance in the experiment and

Domenico Serra for his help with the laboratory analyses.

REFERENCES

- Association of Official Analytical Chemists. 2000. Official Methods of Analysis. 17th ed. AOAC, Gaithersburg, MD.
- Battaccone, G., A. Nudda, A. Cannas, A. Cappio Borlino, G. Bomboi, and G. Pulina. 2003. Excretion of aflatoxin M1 in milk of dairy ewes treated with different doses of aflatoxin B1. *J. Dairy Sci.* 86:2667–2675.
- Battaccone, G., A. Nudda, M. Palomba, and G. Pulina. 2002. Trasferimento di aflatoxina dalla razione al latte ovino e alla cagliata. *Sci. Tecn. Latt. Cas.* 53:283–293.
- Berg, T. 2003. How to establish international limits for mycotoxins in food and feed? *Food Contr.* 14:219–224.
- Dimauro, C., P. Bonelli, N. P. P. Macciotta, P. Nicolussi, C. Patta, and G. Pulina. 2005. Analysis of probability distribution of some serum and haematological variables of dairy sheep. *J. Dairy Sci.* (Abstr.) (accepted).
- Edrington, T. S., R. B. Harvey, and L. F. Kubena. 1994. Effect of aflatoxin in growing lambs fed ruminally degradable or escape protein sources. *J. Anim. Sci.* 72:1274–1281.
- European Commission. 2001. Commission Regulation 2001/466/EC of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs (Text with EEA relevance). *Off. J. Euro. Commun. L.* 77:1–13.
- European Commission. 2003. Commission Directive 2003/100/EC of 31 October 2003 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council on undesirable substances in animal feed (Text with EEA relevance). *Off. J. Euro. Commun. L.* 285:33–37.
- European Communities. 1986. Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. *Off. J. Euro. Commun. L.* 358:1–28.
- Frobish, R. A., D. D. Bradley, D. D. Wagner, P. E. Long-Bradley, and H. Hairston. 1986. Aflatoxin residues in milk of dairy cows after ingestion of naturally contaminated grain. *J. Food Prot.* 49:781–785.
- Galvano, F., V. Galofaro, A. De Angelis, M. Galvano, M. Bognanno, and G. Galvano. 1998. Survey of the occurrence of aflatoxin M1 in dairy products marketed in Italy. *J. Food Prot.* 61:738–741.
- IARC. 2002. Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene. Summary of data reported and evaluation. IARC Monographs on the Evaluation of the Carcinogenic Risk to Humans. Vol. 82. International Agency for Research on Cancer, Lyon, France.
- International Dairy Federation. 1964. Determination of casein content of milk. Standard 29. IDF, Brussels, Belgium.
- International Dairy Federation. 1986. Determination of nitrogen content (Kjeldahl method) and calculation of crude protein content. Standard 20A. IDF, Brussels, Belgium.
- International Organization for Standardization. 1975. Cheese. Determination of Fat Content. Van Gulik Method. ISO Standard 3433. International Organization for Standardization, Geneva, Switzerland.
- Kiermeier, F., and M. Buchner. 1977. On the aflatoxin M1 content of the cheese during ripening and storage. *Z. Lebensm. Unters. Forsch.* 164:87–91.
- Lopez, C., L. Ramos, S. Ramadan, L. Bulacio, and J. Perez. 2001. Distribution of aflatoxin M1 in cheese obtained from milk artificially contaminated. *Int. J. Food Microbiol.* 64:211–215.
- Morris, T. R. 1999. Experimental Design and Analysis in Animal Sciences. CABI Publishing, Oxon, UK.
- Munksgaard, L., J. Larsen, H. Werner, P. E. Andersen, and B. T. Viuf. 1987. Carry over of aflatoxin from cows' feed to milk and milk products. *Milchwissenschaft* 42:165–167.

- Nageswara Rao, S. B., and R. C. Chopra. 2001. Influence of sodium bentonite and activated charcoal on aflatoxin M1 excretion in milk of goats. *Small Rumin. Res.* 41:203–213.
- Pietri, A., T. Bertuzzi, P. Bertuzzi, and G. Piva. 1997. Aflatoxin M1 occurrence in samples of Grana Padano cheese. *Food Addit. Contam.* 14:341–344.
- Polan, C. E., J. R. Hayes, and T. C. Campbell. 1974. Consumption and fate of aflatoxin B1 by lactating cows. *J. Agric. Food Chem.* 22:635–638.
- Roussi, V., A. Govaris, A. Varagouli, and N. A. Botsoglou. 2002. Occurrence of aflatoxin M1 in raw and market milk commercialized in Greece. *Food Addit. Contam.* 19:863–868.
- Veldman, A., J. A. C. Meijs, G. J. Borggreve, and J. J. Heeres-van der Tol. 1992. Carry-over of aflatoxin from cows' food to milk. *Anim. Prod.* 55:163–168.
- Westlake, K., R. I. Mackie, and M. F. Dutton. 1989. In vitro metabolism of mycotoxins by bacterial, protozoal and ovine ruminal fluid preparations. *Anim. Feed Sci. Technol.* 25:169–178.