M4.7, ronchi@unitus.it

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Evaluation of AFB_1/AFM_1 Carry-Over in Lactating Goats Exposed to Different Levels of AFB_1 Contamination

B. Ronchi, P.P. Danieli, A. Vitali, A. Sabatini, U. Bernabucci, and A. Nardone

Dipartimento di Produzioni Animali, Università della Tuscia, via De Lellis, 01100, Viterbo, Italy, e-mail: ronchi@unitus.it

ABSTRACT

A study was performed to assess AFB_1/AFM_1 carry-over in milk of goats exposed to diet with different level of contamination. A 4x4 Latin-square design was used for comparative carry-over trials. Four lactating Saanen goats were housed in metabolic cages. The experimental period lasted 48 d. Each trial lasted 12 d: 7 d treatment sub-period in which AFB_1 was administrated via naturally contaminated corn at four levels ranging from 0 to 57.4±5.1 µg/kg, and 5 d post-treatment period during which no contaminated feed was given. During trials, samples of milk and urine, and feeds and faeces were collected daily and analysed for AFM_1 (milk and urine) and AFB_1 (feeds and faeces) by IAC-HPLC method. Performance of method was routinely checked. AFM_1 concentration in milk was strongly affected ($P<0.01$) by AFB_1 treatment. In contrast subject or trial did not significantly affected AFM_1. Determination of aflatoxins (AF) in feeds, faeces, milk and urine allowed to get interesting results on AF balance. The carry-over of AFM_1, expressed as µg/kg AFM_1 excreted/ µg/kg AFB_1 ingested, was quite high ranging between treatments from 0.029 to 0.031. Results on AFB_1/AFM_1 carry-over in goats are novel, and show that carry-over level for goat is higher than that reported for small ruminants but comparable to levels stated for dairy cows.

INTRODUCTION

Aflatoxins are highly toxic secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus* that may grow on a variety of feedstuffs. Excretion of AFB_1 and its metabolites occurs primarily through the biliary pathway, followed by urinary pathway (Hsieh & Wang, 1994). In lactating animals a consistent fraction of the ingested AFB_1 is excreted into the milk as the hydroxilated form Aflatoxin M_1 (AFM_1) (Allcroft et al., 1966), that has been included in the class 2B by IARC (1993) and considered a potential carcinogenic compound. A lot of studies were conducted on several animal species to establish the amount of AFM_1 excreted into the milk as a percentage of AFB_1

intake (Van der Linde et al., 1964; Petterson et al., 1980; Nageswara Rao & Chopra, 2001; Hoogenboom et al., 2001; Battacone et al., 2003). Those researchers estimated a carry-over ranged from 0 to 4 %. The percentages vary from animal to animal, from day to day and from one milking to the next. In addition, quantities seem to depend on the milk yield and the lactation period (Eaton and Groopman, 1994). For goats milk products, as cheeses, the occurrence of high AFM_1 contamination is reported in a recent survey conducted in southern Italy by Minervini et al. 2001, pointing out that AFM_1 carry-over in this specie may pose a real hazard for consumers health due to high concentrate inputs in dairy goat farming system. Scarce information about milk AFM_1 carry-over in small ruminants is available, particularly in goats. Therefore, the aim of this study was to investigate the AFB_1/AFM_1 carry-over into the milk, and distribution of AFB_1/AFM_1 in urines and feces in goats exposed to different levels of AFB_1 via naturally contaminated corn.

MATERIAL AND METHODS

Animals and experimental design

A 4x4 Latin-square design was used for comparative carry-over trials. Four multiparous Saanen goats in late lactation (average BW 59.2 ± 6.1 kg) housed in metabolic cages were used. The diet consisted of hay ad libitum, 450 g of corn and 450 g of commercial mixed feed. Goats were milked manually at 7:30 AM and 6:30 PM. The experimental period lasted 48 days divided into 4 cycles of 12 days. Each cycle was divided in a 7 days of treatment period followed by a 5 days of post-treatment period. During the treatment period AFB_1 was administrated via naturally contaminated corn at four levels (from 0 to 57.4 ± 5.1 μg/kg; Table 1), whereas during the post-treatment period animals were fed on AFB_1-free corn. Dry matter intake (DMI), milk yield, urine and feces production were recorded daily, whereas body weight (BW) were recorded weekly. Morning milking was blend with evening milking so that one sample was taken daily for each animal and stored a –20°C. Moreover, individual samples of 20 % of total feces and about 5 % of total urine produced were taken daily and stored a –20°C.

Laboratory analytical procedures

Feedstuffs sample preparation – To ascertain the amount of AFB_1 administered to treated animals, corn, commercial mixed feed and hay were submitted to the following analytical procedure: 25 g of dry milled sample was extracted by shaking with 100 ml (corn and commercial mixed feed) and 150 ml (hay) methanol-water (80:20) mixture containing 10% NaCl (p/v). After 30 min, extract was filtered on paper (S&S 589^5 Red Ribbon, Scleicher&Schuell, Dassel, Germany) and then diluted five folds with ultra-pure water (ASTM Type I). Diluted extract was finally filtered on glass fiber filters (Vicam, Watertown, MA, USA). Filtered extract (30 ml) was applied on an Alfatest-P® immuno-affinity column (Vicam, Watertown, MA, USA) and impurities were removed by washing two times the column with 10 ml of ultra-pure water. Alfatoxin B_1 was eluted with 1.5 ml of HPLC grade methanol and collected into microtube. The eluate was evaporated under gentle pure nitrogen stream (Rivoira, Italy) at 37°C. Aflatoxin B_1

in purified extract, was derivatized with 200 µl of trifluoroacetic acid (TFA, Sigma-Aldrich, Seelze, Germany) for 15 min in the dark, then TFA was evaporated and mycotoxin was resuspended in 0.4 ml of methanol-water (45:55, v/v) and transferred to 1.8 ml vial for HPLC analysis.

Milk sample preparation **-** Milk clean-up for Aflatoxin M_1 was performed on skimmed milk, and filtered on microfiber filter. A 30 ml aliquot was passed trough Aflatest P® (Vicam, Watertown, MA, USA) and processed as for feeds but without TFA derivatization.

Urine and feces sample preparation - Urine samples were analyzed after an enzymatic hydrolysis as reported by Kussark et al. (1995). After hydrolysis, 20 ml of sample was cleaned up on the immuno-affinity column Aflatest-P® (Vicam, Watertown, MA, USA) and processed as reported for milk samples. A 25 g sub-sample of dried feces was blended at high speed with 130 ml of methanol-water (80:20) mixture containing 10% NaCl (p/v) for 3 minutes. The slurry was centrifuged at 1500 x g and the supernatant was diluted five folds with ultra-pure water. The diluted supernatant was then filtered on glass fiber and submitted to immunoaffinity clean-up and concentration following the same method reported above for feedstuffs.

RP-HPLC analysis - All cleaned up samples were analyzed and Aflatoxins (B_1 and M_1) quantified by an HPLC system (Thermo separation Products, Riviera Beach, FL, USA) configured as follow: a Membrane Vacuum Degaser SCM 1000, a Spectra-System Gradient Pump P4000, a Spectra-System Autosampler AS3000 with sample tray temperature control (at 0°C) and column oven (at 35°C), and a Spectra-System Fluorescence Detector FL 3000 with an excitation wavelength set at 360 nm and emission wavelength at 440 nm. HPLC system was equipped with a 5 µm, 150 x 4,6 mm I.D. Discovery® C18 main column (Supelco, Bellefonte, PA, USA) and a 5µm Viva C-18, 20 x 4.0 mm guard column (Restek, Bellefonte, PA, USA). Mobile phase, methanol-water (45:55, v/v), was pumped at a flow rate of 1.0 ml/min. Quantification of AFB_1 and AFM_1 was obtained by comparing the injected samples (100 µl) with working solutions containing certified standards of AFB_1 (LGC Promochem, Wesel, Germany) and AFM_1 (Supelco, Bellefonte, PA, USA).

Methods validation - Recovery trials were performed on spiked urine and spiked milk samples (AFM1) and spiked feces; recovery for AFB1 in feeds was estimated processing a certified reference material (CRM n. 371, Commission of the European Community – Community Bureau of Reference) obtained from LabService Analytica (Italy) and treated as reported in Van Egmond et al. (1994). Linearity of the limits of detection (LOD) and limits of quantification (LOQ) were estimated on 7-levels spiked blank samples of milk and corn. Fitting of calibration model was used to check linearity while estimates of LOD and LOQ were obtained on the basis of detector response variability (DIN, 1994) at lower spiking level of 0.005 µgAFM_1/kg and 0.5 µgAFB_1/kg. Linearity and limits for urine and feces were assumed as similar as for milk and feeds.

Statistical analysis

Data for all measured variables were analyzed as repeated measures using the GLM procedure of SAS (SAS, 1999). The model was used to estimate the effects of treatment, sampling day and their interaction. Treatment, sampling day and interaction were statistically significant for all variables tested. The model used was the following:

$Y_{ijkl} = \mu + S_i + T_j + D_k + (T*D)jk + e_{ijkl}$

where:

Y_{ijkl} = dependent variable

μ = overall mean of the population

S_i = mean effect of subject (i = 1,….4);

T_j = mean effect of treatment (j = 1,…4) with day as a repeated factor;

D_k = mean effect of day of sampling (k = 1, ..9);

e_{ijkl} = unexplained residual element assumed to be independent and normally distributed.

RESULTS AND DISCUSSION

Analytical processes

Methods validation results are reported in Table 1. In particular, LOD and LOQ were as good as required on the basis of expected levels of AFB_1 and its metabolites contamination in milk, feces and urine, following the exposure.

Table 1 – Performance of methods for AFB_1-AFM_1 analysis

Matrices	Level	Method recovery	Method LOD-LOQ	Method linearity
Spiked corn (n=3)	0.5 – 64.0*	0.87±0.08 (n=4)	0.12 – 0.38 *	R^2 = 0.9985 (n=3) Range: 0-100*
CRM n. 371 (mixed feed)	9.3±0.5* (certified)	0.78±0.06 (n=3)		
Spiked feces (n=2)	1.0, 2.0*	0.51 – 0.58		
Spiked milk (n=3)	0.005 – 0.320**	0.98±0.08 (n=3)	0.0016 – 0.0050**	R^2=0,9959 (n=3) Range 0-0.160**
Spiked urine (n=2)	0.100, 0.500**	0.66 – 0.73		

*data expressed as $\mu gAFB_1/kg$; ** data expressed as $\mu gAFM_1/kg$

Poor recovery was obtained on spiked feces probably as consequence of limited extraction capacity of methanol-water medium if compared with stronger agent for aflatoxins partition as chloroform (Hoogenbom et al., 2001). No correction for recovery was applied on all analytical data.

Feeds **–** Only corn resulted contaminated by AFB_1 (57.4±5.1 µg/kg AFB_1) and was used as AFB_1 carrier in the diets. Three different levels of exposure were obtained mixing the contaminated corn with a second stock of AFB_1-free corn (Table 2). Average DMI was calculated to be 2.33, 2.38, 2.40 and 2.36 kg/head/day in control, low level, medium level and high level treatments respectively, and differences were statistically non-significant.

Table 2 – AFB_1 contamination of feedstuffs used to prepare experimental rations and diets formulation for different exposure level tested.

	Feedstuffs			
	Contaminated corn stock	AFB_1-free corn stock	Commercial mixed feed	Hay
AFB_1 µg/kg	57.4 ± 5.1 (n=5)	< LOD[¥] (n=3)	< LOD (n=3)	< LOD (n=3)

Treatment (AFB_1 exposure[§])	Contaminated corn (kg)	AFB_1-free corn (kg)	Commercial Mixed feed (kg)	Hay (kg)
Control (**0**) 0.0 µg/day	-	0.45	0.45	2.50
LL[1] (**1**) 9.1 µg/day	0.15	0.30	0.45	2.50
ML[1] (**2**) 18.3 µg/day	0.30	0.15	0.45	2.50
HL[1] (**3**) 27.4 µg/day	0.45	-	0.45	2.50

[¥]LOD=Limit of Detection of method. [§]Nominal exposure values based on mean AFB_1 concentration in contaminated corn stock.
[1] LL = Low Level; ML = Medium Level; HL = High Level

Milk – Milk yield, computed on the whole experimental period, was not statistically significant among treatments (1.23, 1.24, 1.25 and 1.20 kg/head/day for control, LL, ML and HL, respectively). Aflatoxin-M_1 detected in milk, showed maximum concentration for each treatment 48 hours after the administration of contaminated AFB_1 rations (Figure 1). At this time, and during the next 48 hours, differences in AFM_1 concentration in milk compared to the control and between treatments were markedly high ($P<0.05$). During the post-treatment period (formerly from day 8 to day 12) a rapid decrease of AFM_1 level in milk was observed each treatment, leading toward uniformity to lower milk contamination within 48-72 h after the end of exposure. Similar rate of clearance were reported for cows (Frobish et al., 1986), goat (Nageswara Rao & Chopra, 2001) and sheep (Battacone et al., 2003). Moreover, a proportional increase of AFM_1 concentration in milk was observed, ranging from control to higher exposure level tested (Fig. 2); the same findings were reported by Battacone et al. (2003) for dairy ewes fed on an artificial AFB_1 contaminated diet.

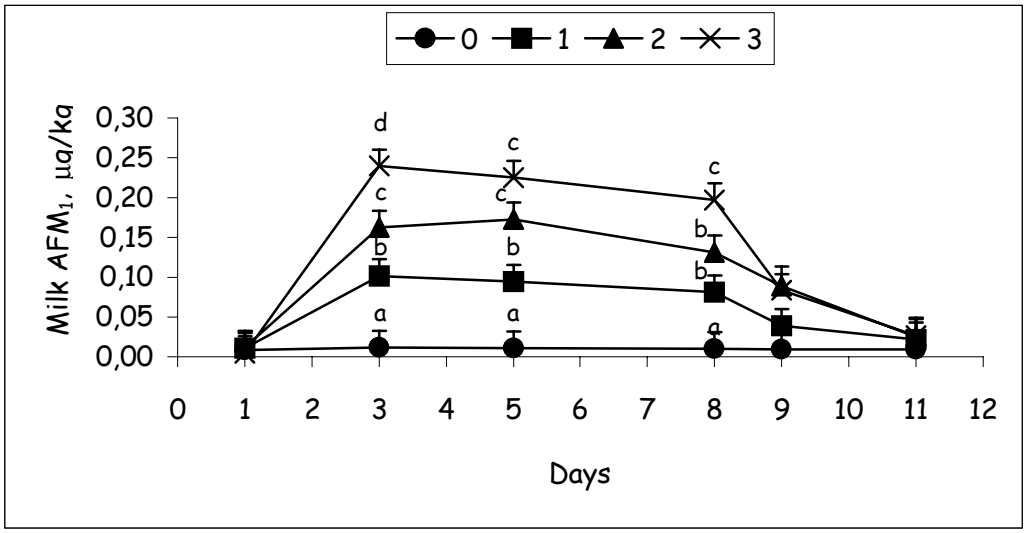

Figure 1 – Ls means ± SE of AFM_1 concentration in milk at different exposure condition (**0** = control, **1 Low Level** = 9.1 µgAFB_1/day; **2 Medium Level** = 18.3 µgAFB_1/day, **3 High Level** = 27.4 µgAFB_1/day). a, b, c, d = *P < 0 .05*

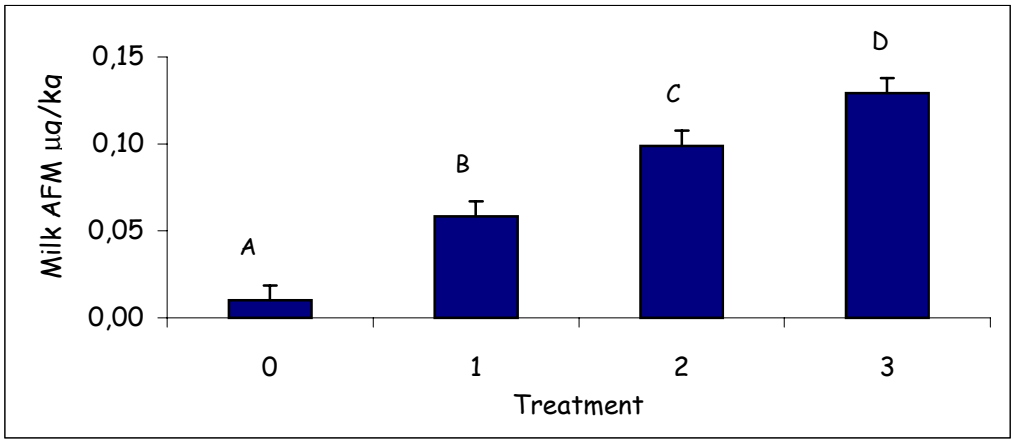

Figure 2 – Ls means ± SE of AFM_1 concentration in milk, mean values and standard error of mean per treatment, calculated on the whole experiment duration (**0** = control, **1 Low Level** = 9.1 µgAFB_1/day, **2 Medium Level** = 18.3 µgAFB_1/day, **3 High Level** = 27.4 µgAFB_1/day). A, B, C, D = *P < 0.01*

The AFM_1 carry-over into the milk, expressed as concentration of AFM_1 on concentration of AFB_1 in daily ration (Figure 3a) and as daily amount of AFM_1 excreted on AFB_1 ingested (Figure 3b), was calculated at day 3 and day 5 after the start of exposure. No significant differences were observed between treatments, although a negative trend between carry-over and AFB_1 exposure was found. Between treatments, concentration of AFM_1 in milk accounted for 2.9-3.1% of the mean diet contamination of AFB_1, whereas a fraction of the amount AFB_1 ingested ranging within 0.80-1.14% was found excreted in milk.

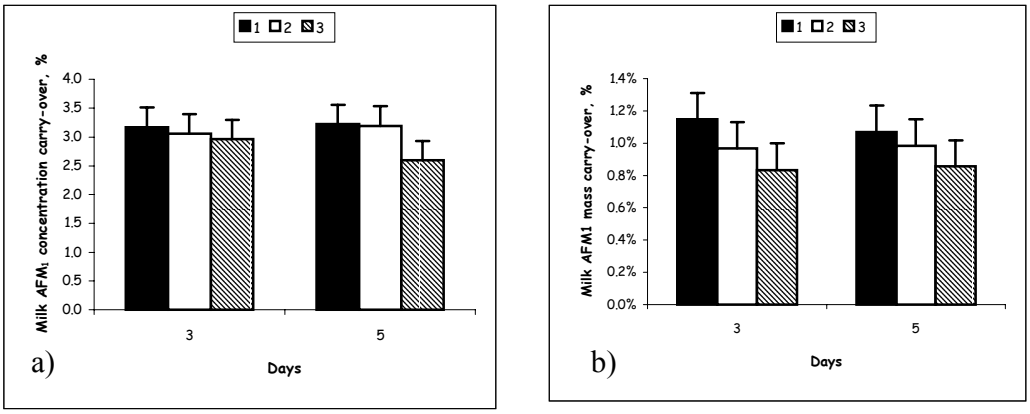

Figure 3 – a) Ls means ± SE of AFM_1 carry-over expressed as AFM_1 concentration in milk on AFB_1 concentration in the diet and b) expressed as $\mu gAFM_1$ daily excreted with milk per $\mu gAFB_1$ daily ingested (**1 Low Level** = 9.1 $\mu gAFB_1$/day, **2 Medium Level** = 18.3 $\mu gAFB_1$/day, **3 High Level** = 27.4 $\mu gAFB_1$/day).

Those findings indicate a passage of AFB_1 metabolites (specifically AFM_1) from feed to goat milk higher than that observed for goat and sheep (Nageswara Rao & Chopra, 2001; Battacone et al., 2003; 2004), but quite similar to what was recently demonstrated for AFB_1 residues in cow milk by Hoogenboom et al. (2001) and within the commonly carry-over values for cattle found in literature (Polan et al., 1979; Applebaum et al., 1982; Price et al., 1985; Frobish, et al., 1986; Fremy et al., 1987; Galvano et al., 1996; Hogenboom et al., 2001). Despite what has been found about AFM_1 excretion in milk, treatment did not show significant effect on carry-over variability ($P < 0.05$). This match with findings reported by Veldman et al. (1992) for dairy cows and by Battacone et al. (2003) for dairy ewes.

Urine and feces – Aflatoxin M_1 was detected and quantified in urine samples at day 1 and day 8. Interactions time x treatment were found (Figure 4a) on AFM_1 excretion in urine, whereas between treatments, only for the medium exposure levels were observed significant differences ($P < 0.05$) on transfer rate as AFM_1 excreted on the amount of AFB_1 ingested (Figure 4b). The amount of AFB_1 extracted from feces samples, showed a good linkage to treatment level, with a net increase of concentration comparing control with higher contaminated diet (Fig. 5). Extracted AFB_1 from feces accounted for 29% (0.289 ± 0.023) of the total daily ingested AFB_1. This is probably due to low efficiency of methanol extraction (see Table 1 for details) as confirmed by Hogenboom et al. (2001), Those researchers, using methanol were able to achieve a maximum percentage of extraction around 46,2% of total AFB_1 residues in cows feces. Moreover, they pointed out AFB_1 derivatives in feces to be as high as 72% of total daily excretion.

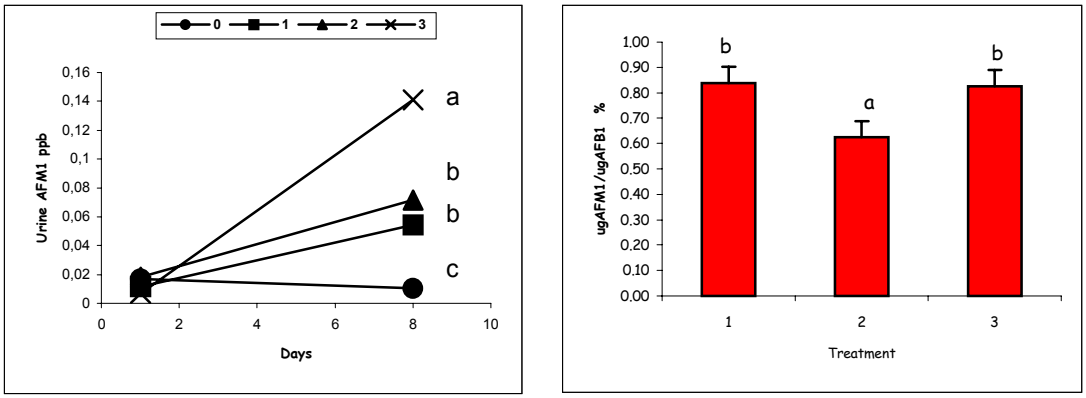

Figure 4 – a) *Time x treatment* interaction on AFM_1 excreted with urine and b) transfer rate expressed as $\mu gAFM_1$ daily excreted with urine per µg of AFB_1 daily ingested. (**0** = control, **1** = 9.1 $\mu gAFdB_1$/day, **2** = 18.3 $\mu gAFB_1$/day, **3** = 27.4 $\mu gAFB_1$/day). a, b, c = *P<0.05*

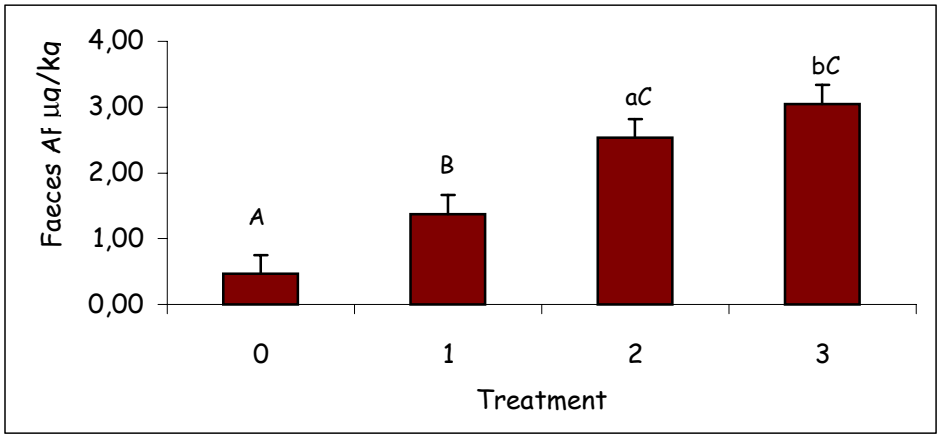

Fig 5 –AFB_1 extracted from feces; data expressed as mean values and standard error of mean per treatment. (0 = control, 1 Low Level = 9.1 $\mu gAFB_1$/day, 2 Medium Level = 18.3 $\mu gAFB_1$/day, 3 High Level = 27.4 $\mu gAFB_1$/day). A, B, C = *P < 0.01*; a, b = *P < 0.05*.

AFB_1 balance – Within the range of AFB_1 exposure levels tested, percentage of AFB_1 and AFM_1 detected and quantified are reassumed in Table 3. Apparent AFB_1 excretion was poor compared to the amount of daily intake. Results were probably influenced by a poor extraction of AFB_1 from feces and maybe from urine. Corrected percentages may be computed using recoveries estimated for all matrices (Table 1).

Table 3 - AFM_1 and AFB_1 excreted with milk, urine and feces at different exposure level. Results are expressed as fraction (%) of the daily intake.

AFB_1 daily intake	Milk AFM_1	Urine AFM_1	Feces AFB_1	Total equivalent AFB_1
9.1 µg	1.14	1.39	31.6	34.1
18.3 µg	0.84	0.97	27.3	29.1
27.4 µg	0.80	1.60	27.7	30.1

Moreover, not all administered AFB_1 and relative metabolites could be detectable into feces, milk and urine; Hogenboom et al. (2001), administering ^{14}C-AFB_1 to cows with diet, were able to quantify the AFB_1 excreted with milk, urine and feces around 91,4% of total ingested radioactivity. The remaining part they stated to be accumulated in several organs and tissues analyzed after the slaughter of animals. At the moment, no data on AFB_1 burden in blood and other tissues or organs are available.

CONCLUSIONS

Results of the present study, suggest to carefully take in account the risk of AFM_1 contamination in milk above law limits, as a consequence of goat exposure to AFB_1 contaminated diets. In particular, the utilization of maize contaminated by AFB_1 near or below the EC regulation limit (20 $\mu gAFB_1/kg$), may pose serious risks for goat milk commercialization and safety of related products. Nevertheless, high excretion rates of AFB_1/AFM_1 with feces and urine, that greatly reduce AFM_1 concentration in milk within 24-48 hours after last AFB_1 ingestion, allows to managing adequately acute events of milk AFM_1 contamination in goat. Further studies would be carry out, especially approached from an epidemiological point of view, to investigate the field scale dimension of these evidences, and the extent of negative effects of feedstuffs AFB_1 contamination on milk quality and safety in goat.

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