



Original Research

A dairy cow model to assess aflatoxin transmission from feed into milk – Evaluating efficacy of the mycotoxin binder Mycosorb®

C.A. Moran^{1*}, H. Kettunen², A. Yiannikouris³, S. Ojanperä², E. Pennala², I.M. Helander² and J. Apajalahti²

¹Alltech France, 25 rue Greffulbe, 92300 Levallois-Perret, France

²Alimetris Ltd, Koskelontie 19 B, FIN-02920 Espoo, Finland

³Alltech Inc., 3031 Catnip Hill Pike, Nicholasville, KY 40356, USA

Summary

A dairy cow model was established to measure the transmission of aflatoxin M1 (AFM1) into milk. Four Ayrshire mid-lactation dairy cows (ranging 590 – 650 kg body weight and averaging 4.5 years of age) were used in a crossover experimental design in which each cow acted as its own control and was subjected to four dietary treatments administered as six 12-day feeding periods followed by a 7-day washout period. Periods I – VI comprised a 7 d of adaptation followed by 5 d of sampling. Period VII was a 7-d washout with sampling in the final two days. From each morning's milking, yield was recorded and sampled for AFM1 analysis by HPLC. Treatments included a negative control, contamination with AFB1 (5 µg of AFB1/kg feed), AFB1 + Mycosorb® (MTB) 10 g/cow/d, and AFB1 + MTB 50 g/cow/d. In response to the negative control, AFM1 concentrations in milk remained below the limit of detection (<5 ng/kg), whereas cows fed the AFB1-contaminated feed had milk AFM1 concentrations ranging from 110 to 230 ng/kg. At the end of the washout period, AFM1 was again undetectable. The dairy cow model was also used to test the efficacy of the yeast-cell-wall-based mycotoxin binder Mycosorb® (MTB) in reducing secretion of AFM1 into milk by cows fed the same AFB1-contaminated diet. When supplemented at 50 g/head/day, MTB significantly reduced ($P < 0.05$) AFM1 secretion into milk with no adverse effects on milk production. The dairy cow model is a sensitive tool for measuring aflatoxin transmission to milk and mycotoxin binder efficacy at 5 µg of AFB1/kg feed. As it is common for on-farm AFB1 concentrations to exceed 5 µg/kg, more research may be warranted to determine the effectiveness of the model at higher AFB1 concentrations.

Keywords: aflatoxin B1: aflatoxin M1: dairy cow model: Mycosorb®: milk: adsorbent: mycotoxin

(Received 2 May 2013 – Accepted 4 November 2013)

Introduction

The presence of toxic dietary substances in cow's milk is strictly regulated by the European Commission under General Food Law (Regulation EC no 178/2002) with official controls in place for feed (Regulation EC no 152/2009) to prevent excessive health burden on the animal and to protect consumers. In particular, these controls apply both to aflatoxin B1 (AFB1), whereby the maximum concentration in feed for dairy cows is restricted to 5 µg/kg complete feed (12% moisture basis) by Directive 2002/32/EC, and to aflatoxin M1

(AFM1) whereby the maximum concentration of AFM1 in milk is restricted to 0.05 µg/kg (*i.e.* 50 ng/kg) (Commission Regulation EU No 165/2010). Aflatoxin is a toxic, mutagenic and carcinogenic mycotoxin produced by the fungal species *Aspergillus*, typically contaminating a wide range of agricultural products including grains, meat and dairy products (Anfossi *et al.*, 2011). Aflatoxin exists in four major forms: B1, B2, G1 and G2 with AFB1 being the most common and most toxic. Aflatoxin M1 (AFM1), a somewhat less toxic

*Corresponding author: cmoran@alltech.com

metabolite of AFB1, is a known carcinogen that contaminates the milk of cows fed AFB1-contaminated feed. Aflatoxin M1 can be detected in milk within hours after administering an oral dose of AFB1 to lactating dairy cows, indicating that at least part of its absorption occurs in the rumen (Moshini *et al.*, 2007). Because up to 6% of AFB1 consumed by lactating dairy cows is secreted into milk as AFM1 (Van Egmond and Dragacci, 2001), affected milk and dairy products entering the food chain can pose a health safety risk (Jouany *et al.*, 2009).

Mycotoxin inactivators, also called mycotoxin adsorbents, binders or sequestering agents, can be used to inhibit the uptake of mycotoxins by the gut. Under Regulation (EC) No. 386/2009, mycotoxin inactivators have been established as a novel feed additive functional group (*m*): ‘substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action’. The yeast cell wall product Mycosorb® (MTB) has been shown to reduce the harmful effects of fungal toxins during digestion by attracting and irreversibly binding them via electrostatic mechanisms in the intestinal lumen, thus quantitatively reducing their uptake (Yiannikouris *et al.*, 2003; Diaz *et al.*, 2004). This binding capability has been demonstrated for dietary concentrations of AFB1 substantially in excess of the EU maximum allowable limits (Diaz *et al.*, 2004; Kutz *et al.*, 2009). However, in the European Union, MTB is officially classified as a feed material (12.2.6 Yeast Product, Regulation EU No. 68/2013) rather than a mycotoxin inactivator. Unlike feed additives that undergo a pre-market scientific evaluation, at the time of this writing, claims for the functional properties of feed materials are not assessed by the European Food Safety Authority (EFSA) (Trunk, 2009; Kavanagh *et al.*, 2013). Nevertheless, the European Commission has ‘clarified that the additive functions as laid down in Annex I of Regulation 1831/2003 are not exclusively for feed additives but can be exerted as well by feed materials’ (SCFCAH, 2010). EFSA provides guidance for the experimental assessment of feed additives with mycotoxin-inactivating properties (EFSA, 2010).

The objective of this study was to evaluate the effectiveness of utilising a dairy cow model to assess the rate of aflatoxin transmission to milk from feed experimentally contaminated with AFB1 within EU regulatory limits. The model was then applied to determine the efficacy of dietary MTB in reducing the concentration of AFM1 secreted in milk.

Materials and methods

The research and animal care protocols were in accordance with European Union guidelines on the protection of animals used for scientific purposes (Directive 2010/63/EU, 2010). Four Ayrshire mid-lactation dairy cows (ranging 590 – 650 kg body weight and averaging 4.5 years of age) were used in a crossover experimental design in which each cow acted as its own control and was each subjected to four dietary treatments administered in a series of six 12-day feeding periods followed by a seven day washout period (Table 1). Negative control treatments were administered twice (periods I and VII), an AFB1 experimentally contaminated treatment (AFB1 diet without MTB) was administered three times (periods II, IV and VI), and the AFB1 experimentally contaminated treatment supplemented with MTB at two dosage levels each was administered once (periods III and V). This design was adopted because a baseline for the AFB1-contaminated diet extending across the entire trial was considered essential to evaluate the model. Cows were housed at a commercial dairy farm within a group of approximately 30 milking cows. They were milked daily at 0600 and 1800 h.

The dietary treatments were fed to each cow individually such that feed intake was 16 kg of dry matter/cow/day, equally divided between compound feed and silage. Each cow received 8 kg of the compound feed (64% oats, 25% wheat, 7.5% barley and 3.5% peas) and had *ad libitum* access to ensiled timothy and meadow fescue, with a moisture content of approximately 35%. The mean daily consumption of silage was 8 kg DM per head providing a calculated AFB1 concentration in the total daily ration intake of 5 µg/kg (DM basis) or 4.4 µg/kg (12% moisture basis).

Pure AFB1 (Sigma-Aldrich, St. Louis, MO, USA) was used to prepare the toxin-contaminated dietary treatments. The toxin was dissolved in 25 ml of methanol

Table 1. Design and the sequence of dietary treatments introduced to dairy cows. Each dietary period I to VI consisted of a seven-day adaptation period followed by a five-day sampling period. The final control period covered days 73 to 79 with sampling on days 78 and 79.

Period	Treatment name	AFB1 µg/kg daily ration	MTB g/cow/d
I	Control	0	0
II	AFB1	5	0
III	+MTB Dose 1	5	10
IV	AFB1	5	0
V	+MTB Dose 2	5	50
VI	AFB1	5	0
VII	Control	0	0

and mixed into 50 g of coarse ground, baking-grade wheat flour. The solvent was evaporated over a 48-h period and the flour and AFB1 thoroughly mixed. The toxin premix was then gradually mixed with the compound feed to yield 5 µg AFB1/kg feed DM basis. The yeast cell wall preparation, MTB (Mycosorb[®], Alltech Inc., Nicholasville KY, USA), was likewise first mixed into wheat flour before mixing into the compound feed to form two dietary treatments delivering 10 g MTB /cow/day (level 1) and 50 g MTB /cow/day (level 2) in periods III and V, respectively (Table 1).

On analysis by LC-MS/MS (PreMervo Analytical Laboratory, Utrecht, The Netherlands), AFB1 in the control feed was below the limit of detection (i.e. <1 ppb). In the analysis of the AFB1-contaminated diet, 73% of the added AFB1 was recovered. In the diets contaminated with AFB1 and containing MTB levels 1 and 2, the recovery of AFB1 in the analysis was 70% and 63%, respectively. The discrepancy between calculated and recovered AFB1 concentrations may in part be attributed to incomplete AFB1 extraction from the feed matrix. It is also possible that some AFB1 underwent degradation in the premix or feed. These anomalies highlight the analytical challenges in accurately monitoring toxin levels in commercial feed samples.

For experimental feeding periods I through VI, seven days of adaptation was followed by five days of milk sampling, as recommended by EFSA guidelines on trial design for dairy cattle (EFSA 2011). The experiment concluded with a seven day washout period (VII), and milk was sampled on the final two days. Daily milk yield per cow was recorded. From the entire volume produced per cow during the morning's milking, two 50 ml samples were taken daily. Samples were immediately frozen and stored at -20°C for AFM1 analysis. Milk from periods II-VI was safely discarded to avoid introducing AFM1-contaminated milk to the food chain.

Milk samples were prepared for AFM1 analysis according to the Vicam Afla M1 HPLC Instruction Manual. This procedure, with slight modifications, was previously used by Dragazzi and Grosso (2001) and Rosi *et al.* (2007). Each 50 ml sample was measured into a test tube and centrifuged at 2000 × *g* for 15 min. The fat layer was separated, discarded, and the remaining skimmed milk was passed through a Vicam Afla M1 affinity column at 1–2 drops/sec. After flushing the column with 20 ml of purified water at 1–2 drops/sec, the column was vacuum dried and AFM1 was eluted into a glass test tube with 1.25 ml of acetonitrile:methanol

(3 : 2) and 1.25 ml of water. The eluate was mixed by vortexing and filtered through a 0.45-µm syringe filter; 40 µl was then analysed for AFM1 using HPLC (high performance liquid chromatography) with fluorescence detection. HPLC separation was performed in the isocratic mode using 25% acetonitrile and 75% water on a Phenomenex Luna C18 column (150 × 4.6 mm, 3 µm). The flow rate was 1.2 ml/min and the run time of 12 min. Detection was performed using an excitation wavelength of 365 nm and an emission wavelength of 445 nm. AFM1 eluted as a single peak at 6.861 min with complete baseline separation. The linearity and repeatability of the method was monitored to ensure accuracy; AFM1 concentrations were shown to have a coefficient of variation (CV) of 1.5%, with a detection limit of 5 ng/kg. The carryover of AFM1 in milk was calculated as the percentage of the AFB1 consumed that was excreted as AFM1 in milk after toxin output in milk reached a steady state (Masoero *et al.*, 2007). To approximate a steady state, AFM1 concentrations were averaged over each five day sampling period. Assessment of the mycotoxin-inactivating properties of MTB was completed in accordance with the EFSA guidance (EFSA, 2010).

Analysis of variance (ANOVA) was used to determine whether there were any significant sources of variation in the data. ANOVA was used with two categorical variables (dietary treatment and animal) and one continuous variable (sampling day). The null hypothesis was that the dietary treatment, animal or sampling day did not cause variation in the data. Dunnett's test was carried out *post hoc*. Significance was declared at $P < 0.05$, whereby P denoted the probability of a treatment to obtain a test value as extreme as the test value obtained using the AFB1 treatment.

Results

The dairy cow model was effective for evaluating the relationship between aflatoxin dietary uptake and secretion into milk. The change in AFM1 secretion rates was both rapid and reproducible in response to dietary concentrations of AFB1, which ranged from undetectable to 5 µg/kg feed. Milk production rate and AFM1 concentration in milk were independent ($r^2 = 0.0002$, $P = 0.898$). No major between-cow differences in milk production were identified, and average milk production of cows 1, 2, 3 and 4 was 20.8, 21.9, 20.8 and 22.4 l/day, respectively. Milk production decreased linearly ($P < 0.0001$) over the lactation period. Therefore,

milk production values were normalised to correspond to the levels produced at the start of the trial. Firstly, the coefficient of linear regression was calculated. The coefficient value indicated how much milk production decreased per day. After that, milk production values were adjusted according to the following equation

$$\hat{m}_t = m_t - k_t$$

where m_t is the observed milk production, \hat{m}_t is the normalized milk production, t indicates the number of days starting from 0 and k is the coefficient value (-0.07) obtained from linear regression. After normalisation, the day effect was no longer significant ($P = 0.472$).

The AFM1 concentrations in milk from individual cows are presented in Table 2 for the feeding periods whereby cows received the negative control diet or diet containing $5 \mu\text{g}$ AFB1/kg feed with no MTB. Feeding the control diet resulted in an AFM1 concentration below the limit of detection (*i.e.* $<5 \text{ ng/kg}$), thereby confirming LC-MS/MS results which indicated no detectable AFB1 present in the control feed. In contrast, feeding periods utilising diets containing $5 \mu\text{g/kg}$ AFB1 without MTB resulted in substantial concentrations of AFM1 in milk, ranging from 110 to 230 ng/kg, with a mean of 154.4 ng/kg over three periods. This mean is three-fold greater than the EU maximum residue level for AFM1 of 50 ng/kg milk, but in agreement with previous research using similar dietary concentrations of AFB1 (Battacone *et al.*, 2009; Britzi *et al.*, 2013). The secretion of AFM1 remained relatively constant over each five day sampling period, exhibiting coefficients of variation (CV) of approximately 10% for all three periods (20 – 24 d, 44 – 48 d, 68 – 72 d). However, between-cow variation was high throughout the experiment, with the average CV varying from 25.6% (d 20 – 24) to 33.3% (d 68 – 72). As the concentration of AFM1 varied

significantly between milk from different animals ($P < 0.0001$), the AFM1 concentration values were normalised to a common scale. This was done by comparing the average concentration values of each animal to the average concentration value of all animals. Individual measurements were then adjusted according to the following equation

$$\hat{c}_{t,j} = c_{t,j} \frac{\sum_{j=1}^4 \sum_{t=0}^T (c_{t,j})}{4 \sum_{t=0}^T (c_{t,j})},$$

where $c_{t,j}$ is the observed AFM1 concentration, $\hat{c}_{t,j}$ is the normalized AFM1 concentration, t indicates the number of day and j indicates the number of animal. After normalisation, the average concentration values of all animals were equal (ANOVA p -value 0.876). Normalisation did not alter the treatment effects since all values, regardless of treatment, were processed in a similar fashion.

The carryover of AFM1 in milk was calculated as the percentage of the AFB1 consumed that was excreted as AFM1 in milk at the time when the toxin output in milk reached a steady state (Masoero *et al.*, 2007). In practice, this meant that the mean of AFM1 results from the five day sampling period was applied. Moreover, the AFM1 secretion rates in cows varied significantly ($P < 0.0001$) such that cow 4 > cow 1 > cow 2 > cow 3, with the average AFM1 concentration in milk from cow 3 being 45% lower than that of cow 4. For two of the cows, the AFM1 concentrations remained fairly constant between the three periods, whereas, for cows 1 and 4, AFM1 concentrations tended to be elevated in the later periods. For this reason the average toxin carryover was lower in the first sampling period (d 20 – 24) compared with subsequent periods (d 44 – 48 and d 68 – 72) at 2.88, 3.18 and 3.2%, respectively (Table 2). From other studies examining the carryover of AFB1 in feed to

Table 2. Secretion of aflatoxin M1 (AFM1) into milk of dairy cows in response to experimental contamination of feed with aflatoxin B1 (AFB1), original data pre-normalisation

Cow #	Control diet (days 1 to 12)	Diet with $5 \mu\text{g}$ AFB1/kg (days 13 to 72)			Control diet (days 73 to 79)
	Days 8 to 12 [†]	Days 20 to 24 [†]	Days 44 to 48 [†]	Days 68 to 72 [†]	Days 78 and 79 [†]
1	<5 (-)	154.4 (10.0)	196.7 (5.6)	175.7 (8.7)	<5 (-)
2	<5 (-)	118.1 (11.8)	124.4 (9.1)	121.9 (13.9)	<5 (-)
3	<5 (-)	114.0 (13.8)	110.8 (16.2)	116.3 (11.1)	<5 (-)
4	<5 (-)	189.7 (2.4)	204.9 (8.4)	225.9 (6.8)	<5 (-)
Mean [‡]	<5	144.1	159.2	159.9	<5
Mean carryover, %	–	2.88	3.18	3.20	–
Mean between-cow CV%	–	25.6	31.4	33.3	–

[†]Mean value of Aflatoxin M1 in ng/kg milk (CV %) based on respective sampling period

[‡]EU Maximum Residue Limit is 50 ng Aflatoxin B1 /kg milk

AFM1 in milk it was reported that, in general, for dairy cows milked twice daily, ingested AFB1 carryover was 1–2% in low-yielding cows (<30 kg milk/day) and up to 6% in high-yielding cows (>30 kg milk/day) (Frobish *et al.*, 1986; Masoero *et al.*, 2007; Britzi *et al.*, 2013). In the current study, using low-yielding, mid-lactation cows, milked twice daily, carryover from ingested AFB1 was approximately 3%. The rate of aflatoxin carryover to milk can be influenced by numerous factors including inter-animal differences in rumen degradation activity, hepatic biotransformation, mammary alveolar cell membrane health, feeding regimens, animal health, and milk yield (Van Egmond, 1989; Jouany and Diaz, 2005; Masoero *et al.*, 2007; Fink-Gremmels, 2008; Volkel *et al.*, 2011; Britzi *et al.*, 2013). The lower carryover in the first period of the current experiment may indicate that a steady state was not achieved between the rate of AFB1 dietary intake and the rate of AFM1 secretion into milk. To achieve steady state may require more than seven days of adaptation or the intake of a threshold quantity of AFB1. In future studies, a longer adaptation period should be considered in applying this model.

Clearance of aflatoxin from milk was rapid, with concentrations undetectable at six and seven days after final administration of AFB1 in diet. This finding is consistent with reports of rapid clearance of AFM1 from milk one to three days after final oral administration of AFB1 (Frobish *et al.*, 1986; Battacone *et al.*, 2009; Britzi *et al.*, 2013).

The results indicate that the dairy cow model was effective in assessing the efficacy of MTB, a commercial mycotoxin binder, in reducing secretion of AFM1 into the milk of cows fed a diet experimentally contaminated with AFB1 at 5 µg/kg feed. Average normalised milk production for cows fed AFB1-contaminated feed with or without MTB is presented in Figure 1. Milk yield of MTB fed cows was slightly, although significantly, higher ($P=0.03$) than yield from cows fed the AFB1 diets. Thus, MTB did not adversely affect milk production.

Concentrations of AFM1 in milk in response to experimental AFB1 concentrations in feed (<0.001 µg/kg and 5 µg/kg) provided an adequate range (5 ng/kg and 110–230 ng/kg, respectively) to differentiate response to the experimental doses of MTB. Compared with the average milk AFM1 concentrations from periods II, IV and VI, AFM1 concentrations were lower (–1% for MTB 10 g/cow/day and –10.3% MTB 50 g/cow/day) when MTB was supplemented in the feed. The effectiveness of

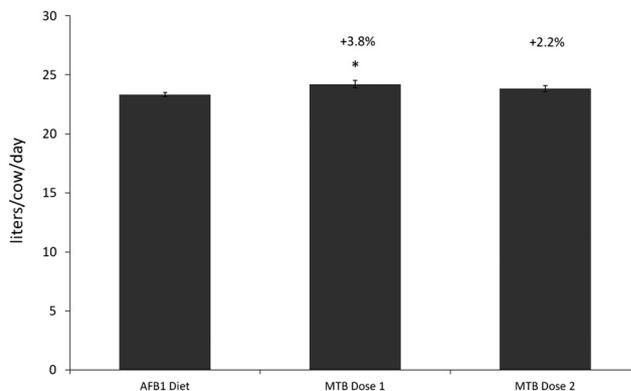


Figure 1. Normalised milk production from cows fed with 5 µg AFB1/kg feed containing no MTB (average of periods II, IV, and VI), MTB at 10 g/cow/d (MTB dose 1) or MTB at 50 g/cow/d (MTB dose 2).

MTB in reducing AFM1 concentration in milk was significant ($P < 0.01$) for the dose of 50 g/cow/day (Figure 2, panel A). As noted previously, AFM1 concentrations in the first AFB1 feeding period (II) most likely never reached a steady state. When AFM1 concentrations from

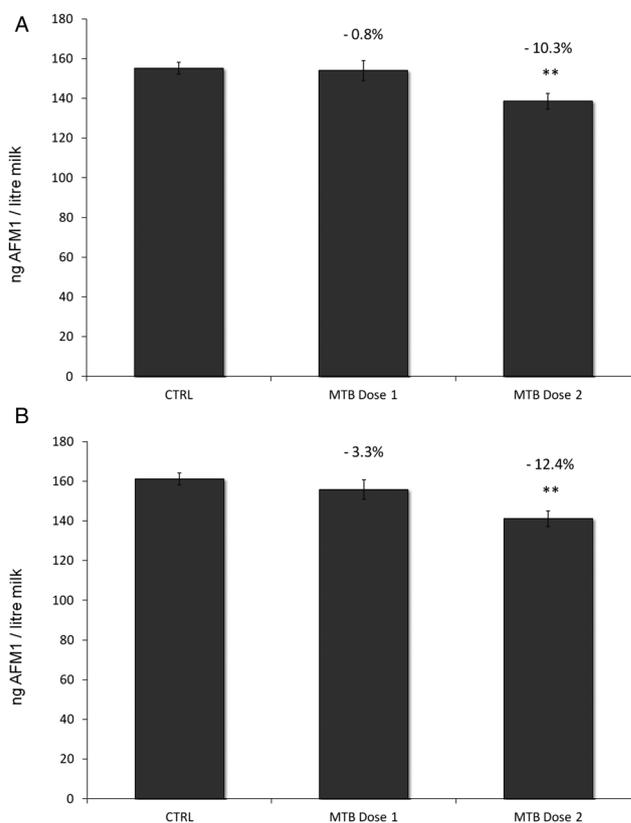


Figure 2. Panel A. Concentrations of AFM1 in the milk from cows fed with 5 µg AFB1/kg feed containing no MTB (periods II, IV, and VI), or MTB at 10 g/cow/d (Dose 1) or MTB at 50 g/cow/d (Dose 2) ($P < 0.01$). Panel B. Concentrations of AFM1 in the milk from cows fed with 5 µg AFB1/kg feed containing no MTB (periods IV and VI), or MTB at 10 g/cow/d (MTB Dose 1) or MTB at 50 g/cow/d (MTB Dose 2) ($P < 0.01$).

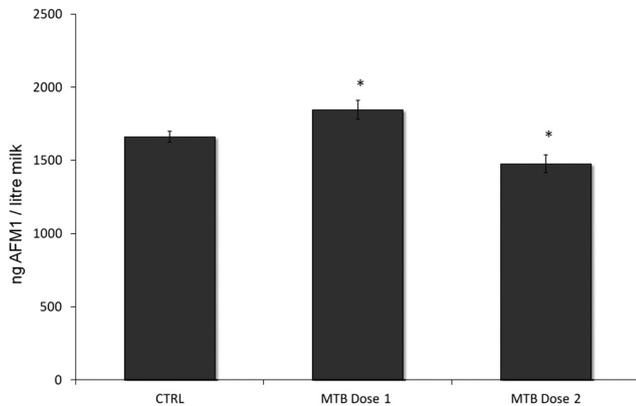


Figure 3. Total AFM1 in morning milk from cows fed with 5 µg AFB1/kg feed containing no MTB (average of periods II, IV, and VI), MTB at 10 g/cow/d (MTB Dose 1) and MTB at 50 g/cow/d (MTB Dose 2).

period II were omitted from the analysis, the reductions in AMF1 concentrations were greater ($P < 0.01$) *i.e.* -3.3% (MTB dose 1) and -12.4% (MTB 50 g/cow/d) (Figure 2, panel B).

Finally, the total amount of AFM1 secreted into daily morning milk was calculated (Figure 3). These results concur with the data presented in Figure 2, where MTB dosed at 50 g/cow/d was shown to significantly ($P < 0.05$) reduce AFM1 concentration. It should be noted that, due to greater milk production by cows supplemented with MTB at 10 g/cow/d, the amount of AFM1 secreted was greater than the amount secreted by cows supplemented with MTB at 50 g/cow/day.

Conclusions

The AFB1 dairy cow model described in the present paper was successfully applied to the assessment of AFM1 secretion into milk by using an initial toxin level of 5 µg/kg feed (DM basis), which is the regulatory action limit allowed for animal trials in dairy cows. To our knowledge this is the first report in dairy cows describing AFM1 monitoring at the EU regulatory action threshold of AFB1. In addition, the effect of 50 g MTB per head per day reduced toxin secretion into milk was demonstrated here at the allowed dietary level of 5 µg AFB1/kg feed (DM basis). The model described has proven a powerful tool for such monitoring. In farm situations, toxin levels are seldom analysed and in many cases likely to exceed the level approved by authorities for animal trials. It is thus possible that mycotoxin binders function even more effectively in actual situations involving feedstuffs with higher mycotoxin contamination.

Acknowledgments

The authors would like to thank Alltech Inc (Lexington, KY, USA) for their financial support of this research.

Declaration of interest

The authors C. A. Moran and A. Yiannikouris are employees of Alltech. Alltech Inc. (Lexington, KY, USA) fully funded this research and own the rights to the mycotoxin binder Mycosorb[®] used in the experiment.

References

- Ansolfi L., Baggiani C., Giovannoli C. & Giraudi G. (2011) Occurrence of Aflatoxin M1 in Dairy Products, in: Torres-Pacheco I. (Ed.) *Aflatoxins – Detection, Measurement and Control*, ISBN:978-953-307-711-6, InTech, Available from: <http://www.intechopen.com/books/aflatoxins-detectionmeasurement-and-control/occurrence-of-aflatoxin-m1-in-dairy-products>
- Battacone G., Nudda A., Palomba M., Mazzette A. & Pulina G. (2009) The transfer of aflatoxin M1 in milk of ewes fed diet naturally contaminated by aflatoxins and effect of inclusion of dried yeast culture in the diet. *J. Dairy Sci.* **92**: 4997–5004.
- Britzi M., Friedman S., Miron J., Solomon R., Cuneah O., Shimshoni J.A., Soback S., Ashkenazi R., Armer S. & Shlosberg A. (2013) Carry-over of aflatoxin B1 to aflatoxin M1 in high yielding Israeli cows in mid- and late- lactation. *Toxins*, **5**: 173–183.
- Diaz D.E., Hagler W.M. JR., Blackwelder J.T., Eve J.A., Hopkins B.A., Anderson K.L., Jones F.T. & Whitlow L.W. (2004) Aflatoxin binders II: reduction of Aflatoxin M1 in milk by sequestering agents of cows consuming Aflatoxin in feed. *Mycopathologia*, **157**: 233–241
- Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed (2002) *Official Journal of the European Union* OJ L 140/10
- Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes *Official Journal of the European Union* OJ L 276/33.
- Dragacci S., Grosso F. & Gilbert J. (2001) Immunoaffinity column cleanup with liquid chromatography for determination of Aflatoxin M1 in liquid milk: collaborative study. *Journal of the AOAC International*, **84**: 437–443
- EFSA (2010) EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Statement on the establishment of guidelines for the assessment of additives from the functional group ‘substances for reduction of the contamination of feed by mycotoxins’. *EFSA Journal* **8**: 1693. [8 pp.]. doi:10.2903/j.efsa.2010.1693.
- EFSA (2011) EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Technical guidance: Tolerance and efficacy studies in target animals. *EFSA Journal* **9**: 2175. [15 pp.]. doi:10.2903/j.efsa.2011.2175.
- Fink-Gremmels J. (2008) Mycotoxins in cattle feeds and carry-over to dairy milk: A review. *Food Additives & Contaminants: Part A*. **25**: 172–180.
- Frobish R.A., Bradley B.D., Wagner D.D., Long-Bradley P.E. & Hairston H. (1986) Aflatoxin residues in milk of dairy cows after ingestion of naturally contaminated grain. *J. Food Protect.* **49**: 781–785.
- Jouany J.P. & Diaz D. (2005) Effects of mycotoxins in ruminants. Pages 295–321 in *The Mycotoxin Blue Book*. D.E. Diaz, ed. Nottingham University Press, Nottingham, UK.

- Jouany J.P., Yiannikouris A. & Bertin G.** (2009) Risk assessment of mycotoxins in ruminants and ruminant products. *Options Méditerranéennes, A* **85**: 205–224.
- Kavanagh G., Pentieva K., Kennedy J. & Moran C.A.** (2013) The impact of Regulation (EC) 767/2009 on the practice of feed advertising in Europe. *Journal of Applied Animal Nutrition*, Vol. 1; e4; page 1 of 10
- Kutz R.E., Sampson J.D., Pompeu L.B., Ledoux D.R., Spain J.N., Vázquez-Añón M. & Rottinghaus G.E.** (2009) Efficacy of Solis, NovasilPlus, and MTB-100 to reduce aflatoxin M1 levels in milk of early to mid lactation dairy cows fed aflatoxin B1. *J Dairy Sci.* **92**: 3959–63.
- Masoero F., Gallo A., Moschini M., Piva G. & Diaz D.** (2007) Carryover of aflatoxin from feed to milk in dairy cows with low or high somatic cell counts. *Animal* **1**: 1344–1350.
- Moschini M., Masoero F., Gallo A. & Diaz D.** (2007) Mucosal absorption of aflatoxin B1 in lactating dairy cows. *Italian Journal Of Animal Science* **6** (Suppl. 1): 324–326.
- Regulation (EC) no 178/2002** of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety (2002) *Official Journal of the European Union*, OJ L 31/1
- Regulation (EC) no 1831/2003** of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition (2003) *Official Journal of the European Union*, L 268/29
- Regulation (EC) No 152/2009** of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. (2009) *Official Journal of the European Union* L 54/1
- Regulation (EC) No 386/2009** of 12 May 2009 amending Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the establishment of a new functional group of feed additives (2009) *Official Journal of the European Union* OJ L 118/66
- Regulation (EC) No 767/2009** of the European Parliament and of the Council of 13 July 2009 on the placing on the market and use of feed, amending European Parliament and Council Regulation (EC) No 1831/2003 and repealing Council Directive 79/373/EEC, Commission Directive 80/511/EEC, Council Directives 82/471/EEC, 83/228/EEC, 93/74/EEC, 93/113/EC and 96/25/EC and Commission Decision 2004/217/EC (2009) *Official Journal of the European Union* OJ L 229/1.
- Regulation (EU) No 165/2010** of 26 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. (2010) *Official Journal of the European Union* L 50/8.
- Regulation (EU) No 68/2013** of 16 January 2013 on the Catalogue of feed materials. (2013) *Official Journal of the European Union* L29/1.
- Rosi P., Borsari A., Lasi G., Lodi P., Galanti A., Fava A., Girotti S. & Ferri E.** (2007) Aflatoxin M1 in milk: Reliability of the immunoenzymatic assay. *International Dairy Journal* **17**: 429–435.
- SCFCAH** (2010) Summary record of the Standing Committee on the Food Chain and Animal Health held on 14 & 15 December 2010 in Brussels. European Commission – DG SANCO. http://ec.europa.eu/food/committees/regulatory/scfcah/animalnutrition/sum_14151220_10_en.pdf p. 1–8.
- Trunk W.** (2009) Better regulation, simplification and modernisation of the EU-legislation in the area of food safety. *European Food and Feed Law Review*, **2**: 130–134.
- Van Egmond H.P.** (1989) Aflatoxin M1: occurrence, toxicity, regulation. In *Mycotoxins in dairy products* (ed. HP van Egmond), pp. 11–55. Elsevier Applied Science, London, UK.
- Van Egmond H.P. & Dragacci S.** (2001) Liquid chromatographic method for aflatoxin M1 in milk, in: Trucksess M.W & Pohland A. E: (Eds.), *Methods in Molecular Biology*, Vol. 157 *Mycotoxin Protocols II*, pp. 59–69 (Totowa, Humana Press).
- Völkel I., Schröer-Merker E. & Czerny C-P.** (2011) The carry-over of mycotoxins in products of animal origin with special regard to its implications for the European food safety legislation. *Food and Nutrition Sciences*, **2**: 852–867.
- Yiannikouris A.L., Poughon X., Cameleyre C.-G., Dussap J., François G. Bertin, & J.-P. Jouany.** (2003) A novel technique to evaluate interactions between *Saccharomyces cerevisiae* cell wall and mycotoxins: application to zearalenone. *Biotechnology Letters* **25**: 783–789.