Aflatoxins M1 and M2 in the milk of donkeys fed with naturally contaminated diet

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Abstract For its nutritional composition, donkey milk is an excellent alternative to breast milk for infants suffering from cow's milk allergies. Even in donkeys could be exist a passage of aflatoxin from contaminated feed to milk as is reported by many authors in other dairy species, but there are no studies about this topic.

This work was aimed at studying the excretion of aflatoxin M1 (AFM1) and M2 (AFM2) in milk after feeding trials with contaminated feed. Six donkeys, at the end of lactation, received a diet with naturally contaminated corn containing 202 and 11 µg/kg of aflatoxin B1 (AFB1) and aflatoxin B2 (AFB2), respectively. Individual milk samples were analysed for AFM1 and AFM2 for 15 days after the contaminated feed administration. Amounts of AFM1 and AFM2 were found in the milk. The steady-state condition was reached after 6 days. No AFM1 or AFM2 were detected in milk after 28 hours from the last contaminated feed administration. The carry-over from AFB1 to AFM1 and from AFB2 to AFM2 was found to be 0.02 and 0.31% respectively.

The results obtained in this study are thus a further step towards understanding the possible carry-over of aflatoxin in donkey milk.

Key Words Aflatoxins · Donkey · Carry-Over · Milk

1 Introduction

Donkey milk, with its natural and healthful properties, is unique as an optimal substitute for infants allergic to cows' milk and for the elderly. The presence of aflatoxins in animal products poses a serious risk to consumers' health. Even in donkey' milk, as in the milk of other species may be present aflatoxins from feed. However in donkeys, have not been carried out studies to investigate the carry-over of aflatoxins in milk from feed to evaluate the associated risk.

Aflatoxins (AFs) are one of the best known and widely investigated groups of mycotoxins which can be found as contaminants in food commodities worldwide. AFs are mainly produced by *Aspergillus* species in agricultural products such as grains, oil seeds, beans, nuts, and dried fruits (Ardic et al., 2008; Decastelli et al., 2007) as well as in hays and ensiled forages. Besides their acute toxicity, mainly targeted at the liver, aflatoxins are well known carcinogenic, teratogenic and mutagenic compounds. In particular, aflatoxin B1 (AFB1), B2 (AFB2), G1 and G2, together with the main oxidative metabolite aflatoxin M1, have been classified as Group I human carcinogens by IARC (IARC, 2002).

After ingestion, AFB1 and AFB2 are metabolized by the liver to their hydroxylated metabolites M1 (AFM1) and M2 (AFM2), that can be excreted in urine, faeces, transferred to milk and, to a lesser extent, to meat (Fink-Gremmels, 2008). Although the AFB1 to AFM1 conversion ratio in milk is rather low (1 – 3%), milk contamination is of concern due to the key role played by milk and dairy products in children's diet (López et al., 2003, Herzallah, 2009). For this reason, the European Union has set at 0.05 µg/kg the limit for AFM1 in milk, and at 0.025 µg/kg for AFM1 in infant formulae and follow-on formulae, including infant milk and follow-on milk [Commission Regulation (EC) N. 1881/2006]. Furthermore the Commission Regulation (EU) N. 574/2011 has set the limit for AFB1 at 20 µg/kg for feed materials, 10 µg/kg for complementary and complete feed and 5 µg/kg for compound feed for dairy cattle and calves, dairy sheep and lambs, dairy goats and

kids, piglets and young poultry animals. No limits are set for AFM2 in milk, while maximum levels have been established in feed for the total amount of aflatoxins.

Donkey milk is quite marginal, and is mostly produced for cosmetic use or as substitute to human milk in European small-scale farms that have chosen diversified production (Jirillo et al., 2010). Donkey with horse and yak produce less than 0.1% of milk from all species including cattle, but no world specific statistics are available (Faye and Konuspayeva, 2012). However today the consumer is increasingly interested in donkey milk and this product is living a phase of success and growth. In the matrix BCG (Boston Consulting Group) this milk is among the products "Question Mark", characterized by low market share and by a high potential of development. Actually donkey's milk is a niche business with high commercial value (Moruzzo and Rossignoli, 2013).

Donkey feed is based on forage, thus to increase donkey milk production it is necessary to supplement the diet with energy sources such as cereals which, in particular climatic condition, are the main target of Aflatoxin B1 and B2.

While for lactating ruminants, such as cows, sheep and goats, a large literature exists, in the case of lactating donkeys very little is known on the occurrence and the transfer of AFB1 and AFB2 as AFM1 and AFM2 from feed into milk.

Kos et al. (2013) investigated the occurrence of AFM1 in milk of different species in Serbia. In particular, the authors detected AFM1 in the milk produced by 3 donkeys, but no information was available about the AFB1 amount ingested by the animals. Very recently, Bilandzic et al. (2014) analysed 14 samples of donkey milk from Serbia, finding an average contamination of 4.77 ± 1.33 ng/L. As milk samples were taken in the summer and AFM1 levels in donkey's milk were lower than in cows' milk, the authors speculated about the availability of pastures and forage for animals during summer.

Because of the lack of knowledge on the carry-over of aflatoxins B in donkey milk, an experiment was conducted to study the relationship between AFB1 and AFB2 intake and AFM1 and AFM2 excretion in donkeys fed naturally contaminated corn.

2 Material and Methods

Six Romagnola breed donkeys at the end of lactation (average 218 days in milking) were used. Daily milk yield and body weight (BW) averaged 1.9 ± 0.3 l and 259.7 ± 6.62 kg (mean ± SD) respectively.

Before the trial started and during the experimental period the health of the animals was monitored continuously. Body weights of donkeys were measured before and at the end of the trial.

The experiment was conducted in accordance with the guidelines of the EC Council Directive (86/609/EEC).

Each animal was fed 8 kg of hay, 0.5 kg of mixed flakes and 1 kg of corn for 25 days. The hay and mixed flakes administered during the trial were not contaminated by aflatoxins. The corn administered during the adaptation period and the last three days of the trial was not contaminated by AFs. After a 10 days' adaptation period, until 22nd day, the corn was replaced with corn naturally contaminated (CNC) by 202 µg/kg of AFB1 and 11 µg/kg of AFB2. During the experimental period the CNC was individually administered by a bucket in the morning. The animals were monitored in order to ensure that the CNC was completely ingested.

Individual milk samples were collected at -24, 0, 1, 4, 8, 24, 32, 48, 56, 80, 104, 128, 152, 224 and 296 hours after the first contaminated corn administration. At the 22nd day the animals were fed for the last time with CNC and individual milk samples were collected at 0, 4, 8, 16, 20, 24, 28, 32, 48 and 72 hours after the last administration. Milk samples were stored at -20°C until the analyses for AFs had been performed.

2.1 Chemicals and reagents

The AFM1 reference standard was purchased by Sigma-Aldrich (Milan, Italy). The AFM2 reference standard was purchased by Fermentek (Jerusalem, Israel). HPLC grade water and solvents were purchased from VWR-PBI (Milan, Italy). The immunoaffinity columns (IAC) IClean C Afla were provided by Tecna Srl (Trieste, Italy). A commercial solution of AFB_1, AFB_2, AFG_1 and AFG_2 in methanol, 1000 ng ml^{-1} was purchased from Aflastandard, R-Biopharm, Madrid, Spain.

All other reagents are analytical gradient.

2.2 Determination of AFM1 and AFM2

Milk analysis was performed by the HPLC-FLD method according to Meucci et al. (2010), slighty modified. Milk samples were previously skimmed by means of the addition of Celite (Sigma-Aldrich) and centrifugation at 3000 rpm for 10 min. After elimination of the fat layer, 50 ml of milk was filtered through Whatman filter paper (Whatman International, Maidstone, UK) and then passed through IAC at a flow-rate of one to two drops per second. The column was washed with 20 ml of PBS (Phosphate Buffered Saline) (one to two drops per second). Elution was performed with 3 ml of methanol. The eluent was evaporated under stream of nitrogen till it became dry. The residue was re-dissolved in 250 µl of HPLC mobile phase and injected into the HPLC system.

The chromatographic system consisted of a Jasco880 pump and a Jasco821 fluorescence detector (Jasco, Tokyo, Japan). JascoBorwin software was used for data processing. The excitation wavelength and emission wavelength were set at 365 and 435nm. The reversed-phase column was a Luna C18 ODS2, 3 µm, (4.6X150 mm) (Phenomenex, Torrance, CA, USA). The column was kept at room temperature. The HPLC was operated with a mobile phase system consisting acetonitrile–water 23/77% v/v at a flow rate of 1 ml/min.

2.3 Confirmation of AFM2 occurrence in milk by LC-MS/MS

The chromatographic analysis for milk samples revealed the presence of another peak related to AFM1. In consideration of the specificity of the analytical protocol and the possible cross-reaction with immunoaffinity antibodies, the peak was tentatively identified as AFM2.

Confirmation of AFM2 identity in milk has been performed by HPLC-MS/MS according to Biancardi et al. (2013) slighty modified. Milk samples were centrifuged at 1006 m/s^2 for15 min and the fat layer discharged. A total of 20 g of the defatted milk was added to sodium chloride (2 g) and ethyl acetate (20 ml). The mixture was stirred for 10 min, then centrifuged at 447 g for 5 min. A total of 5 ml of the organic layer was evaporated under nitrogen flow (at 40°C) and the residue was re-dissolved in 250 µl of formic acid 0.1%:acetonitrile 90:10 (v/v), vortexed and sonicated for 5

min. After centrifugation at 1006 g for 10 min, 170 µl of the solution were transferred in a vial for the LC analysis.

LC-MS/MS analysis was performed with a 6430 Triple Quad MS (Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray interface and a 1290 Infinity separation module (Agilent Technologies). The separation was achieved on a ZORBAX SB-C18 (Agilent Technologies; 50 mm, 2.1 mm I.D., 1.8 mm) column with a pre-column filter (0.3 µm). Gradient elution was performed using eluent A, 0.1% formic acid in water, and eluent B, 0.1% formic acid in acetonitrile. The gradient was set as follows: the first step was a linear gradient from 95% A to 95% B in 5 min, followed by a 1-min isocratic step at 95% B. The column was reconditioned at 95% A for 1.5 min. The total run time was 7.5 min. The following parameters were set: flow rate 0.4 ml/min, column temperature 40°C, injection volume 10 ml.

2.4 Determination of Aflatoxins in feed

Feeds analysis was performed by the HPLC coupled to Kobra cell method according to Hernandez-Martınez and Navarro-Blasco (2010), slighty modified. Feeds (50 g) were carefully weighed and mixed with 4 g of sodium chloride and 250 ml of extracting agent acetonitrile/water (60 : 40, v/v) into a blender jar. After crushing and mixing for 2 min at high speed, the extract was filter through Whatman No. 4 filter paper (Whatman International, Maidstone, UK). A 25-ml volume of filtrate was evaporated (Buchi R-3000 Rotavapor; Büchi Labortechnik AG, Postfach, Switzerland) for 8 min at a temperature of 30°C and a rotation speed of 65 rpm. The evaporation residue was transferred into 50 ml volumetric flasks and topped up with 500 µl acetonitrile and a solution of PBS. Finally, 10 ml of the reconstituted extract were passed through the immunoaffinity column (Tecna, Trieste, Italy) at a flow rate of 2 ml min^{-1}. The column was washed with two aliquots of 10 ml ultrapure water at a flow rate of 5 ml min^{-1}, and the aflatoxins were slowly released from the antibody using 1 ml of methanol and eluted with 1 ml ultrapure water. Finally, the eluted samples were filtered via a PVDF syringe filter (13 mm, 0.22 µm; Tecnokroma, Barcelona, Spain), and collected in vials for HPLC analysis

The chromatographic system consisted of a Jasco880 pump and a Jasco821 fluorescence detector (Jasco, Tokyo, Japan). JascoBorwin software was used for data processing. A Kobra Cell system (R-Biopharm) was used for post-column derivatization. Separation was carried out on a Luna C_{18} column of 4.6 × 150 mm, 5 µm particle size, 100 Å (Phenomenex, Torrance, CA, USA) protected by a pre-column (Phenomenex): precolumn holder, analytical guard cartridge system (4.6 × 10 mm), cartridge guard column and C_{18} cartridge security guard (4 × 3 mm).

2.3 Milk analysis

Milk samples were analysed at the start and at the end of the trial for fat, protein and lactose with a Milkoscan 605 (Foss Electric, Hillerød, Denmark), for somatic cell count (SCC) with a Fossomatic 360 (Foss Electric) and for total microbial count (TMC) with a Bactoscan 8000 (Foss Electric).

2.4 Blood analysis

Blood samples were collected from each donkey by jugular venepuncture before and after the trial for immediate analysis. Haematocrit (HCT) was determined with a Baker System instrument (9120 AX, Biochem Immunosystems Italy).

Serum samples were analysed (Photometer 4010 Boehringer Mannheim, Germany) with colorimetric commercial kits (Roche Diagnostics, Mannheim, Germany) for total creatinine, bilirubin, aspartate amine transaminase (AST), alanine amine transaminase (ALT), glutamic gamma transaminase (GGT), lactate dehydrogenase (LDH).

2.5 Carry-over calculation

The carry-over was calculated as individual daily ratio between the AFM1 excreted in milk and the corresponding AFB1 intake, during the time in which the steady-state output of toxin in milk was reached. The carry-over of AFM2 after AFB2 intake was also performed.

Following as reported by Littell et al. (1998) the steady-state period was established from the first hour of sampling that was not significantly different ($P>0.10$) from average of subsequent hours for each Aflatoxin, within the time of administration of CNC.

2.6 Statistical Analyses

The milk and blood parameters and the AFM1, AFM2 content in milk (to establish the beginning and the duration of the period of steady state output of these aflatoxins in milk) were performed by repeated measure analysis (ANOVA) test using JMP® (Statistical Analysis System Institute, 2007).

3 Results

All the donkeys employed no showed obvious adverse effects on health during the experimental period.

All animals daily ingested the total administered ration. At the end of the trial the donkey body weight was 260.16±7.34 kg (mean±SD). The milk composition as well as SCC and TBC content are reported in Table 1. The blood parameters related to liver function and hematocrit measured at the beginning and at the end of the trial are reported in Tables 2 and 3 respectively.

No AFM1 and AFM2 were detected in the milk before the CNC administration. Both AFM1 and AFM2 were detected in milk of all animals from 1 h after the first CNC administration (Figure 1). During the CNC administration period, in all animals AFM1 and AFM2 content increased in milk from 0 to 104 h (32.25 ng/L) and to 128 h (23.33 ng/L) respectively (Figure 1). Then, AFM1 and AFM2 contents decreased and reached a steady-state condition in the interval between 150 and 296 h. Finally, in the clearance period, AFM1 and AFM2 quickly decreased, and they were no longer detected at 28 h post withdrawal.

The AFM1 and AFM2 carry-over, observed during the steady state, proved to be 0.02% ±0.005 and 0.31% ±0.020 (means ± SE), respectively.

Low levels of AFB1 (1-5 ng/L) were also found in 6 samples of milk.

4 Discussion

The BW was not negatively affected by the presence of AFs in the diet, and remained constant over the observation period.

Fat, protein and lactose concentrations (Table 1) did not vary significantly with AFs ingestion. The SCC content was not influenced by AFs intake, as previously observed in cows (Applebaum et al., 1982), and was comparable with the normal values reported in lactating donkeys

by Colavita et al. (2011). The total bacterial cell count was also under the threshold (1,500,000/ml) (Reg. EC 853/2004).

Similarly, statistical analysis underlined that the aflatoxins intake did not affect significantly the serum parameters (Table 2). As serum enzyme activities are generally elevated in aflatoxicosis as a consequence of hepatocyte damage (Pier, 1992), this result suggests that administration of AFB1 and AFB2 by feeding in this trial did not cause significant hepatic damage. No effects due to aflatoxin were observed for all haematological parameters considered and they were within the normal range (Table 3). The presence of AFM1 in milk was detectable from the first milking (1 h after the AFB1 administration) (Figure 1), which is in accordance with a previous study in goats (Mazzette et al., 2009). Battacone et al. (2003) detected AFM1 in ewes' milk after 6 h from the AFB1 administration. This suggests that the absorption of AFB1 in the gastrointestinal tract and its liver oxidation were very quick processes, as previously found in dairy cows by Moschini et al. (2007), who detected AFB1 and AFM1 in plasma as early as 15 minutes after the ingestion of feed contaminated by aflatoxins.

AFM2 was also detected in donkeys' milk at the first milking (1 h) from the first ingestion of AFB2 contaminated feed (Figure 1).

The highest value of AFM1 concentration in milk (32.25 ng/L) was below the European Community maximum allowed in AFM1 milk levels for adult consumption (50 ng/L) but above the AFM1 milk levels allowed for infants (25 ng/L). After 6 days of CNC daily administration, AFM1 concentration decreased and reached a steady-state condition from 152 h. The trend of AFM1, a first period with high presence of AFM1 in milk followed by a steady-state phase with lower values, has also been found in similar trials by Battacone et al. (2003) on sheep, by Ronchi et al. (2005) on lactating goats and by Bertuzzi et al. (2003) on lactating sows.

The AFM2 content in milk showed a similar trend in comparison with AFM1 (Figure 1). The AFM2 concentration at 32, 56, 104 and 128 h was more than 20 ng/L. These values proved similar to those found for AFM1, although the AFB2 in the CNC administered was 18 times less

than the AFB1 (11 *vs* 202 µg/kg). Also other authors have reported a higher passage of AFM2 in milk of sows and buffalos (Bertuzzi et al., 2003; Pietri et al., 2003).

In the clearance period AFM1 and AFM2 quickly decreased, and disappeared completely after 28 hours after the end of CNC administration (Figure 1). Similar results were found in sows (Bertuzzi et al., 2003). The total disappearance of AFM1 from the last AFB1 administration seems to be faster in monogastric species than ruminants. In fact in dairy cows and in small ruminants disappearance time were about 3 – 4 days from the last AFs administration (Ronchi et al., 2005; Battacone et al., 2003; Masoero et al., 2007).

The AFM1 and AFM2 trend and the disappearance rate in milk were similar. This is probably related to the similar molecular structure. Pietri et al. (2003) and Bertuzzi et al. (2003) carried out trials on buffalos and sows found that AFM1 and AFM2 disappeared from the milk at the same time.

In our study the carry-over of AFB1 in milk (0.02%) was lower compared with that found in other species that ranged from 0.06% in sow, 0.2% in buffalo, 1.8% in goats, 2.90% in sheep to 6.6% in cows (Bertuzzi et al., 2003; Pietri et al., 2003; Ronchi et al., 2005; Battacone et al., 2009; Britzi et al., 2013). The AFM1 carry-over appears to be clearly influenced by the animals species, in particular the carry-over in monogastric animals seems to be lower than that found in ruminants. Ruminants are generally be considered more resistant than monogastric to the adverse effects of mycotoxins, because of the rumen and its microbial population should play a role in detoxification. Ruminal and hepatic bioconversions of mycotoxins alter the polarity of toxins and their affinity to water or lipids, consequently ruminants excretion of toxins and their metabolites in milk results higher then monogastrics (Yannikouris and Jouany, 2002; Jouany et al., 2009).

The carry-over of AFM2 was about 15 times higher than that found with AFM1. This result was in agreement with previous trials in buffaloes (Pietri et al., 2003) and in sows (Bertuzzi et al., 2003).

The higher rate of passage in milk of AFM2 may be related to the lower amount of AFB2 present in contaminated feed. An inverse correlation was observed between AFB1 administered by feeding and AFM1 excreted in sheep and goats milk (Battacone et al., 2009; Ronchi et al., 2005; Nageswara Rao and Chopra, 2001).

The aflatoxin M2 result suggests that careful consideration should be given to the presence of this aflatoxin, which is not regulated by the law in all species of dairy mammals. The level of toxicity of AFM2 could be less than that of AFM1. This may be due to the lack of the double bond terminating the difuran ring system which is common in AFB1, AFG1 and AFM1 (Weindenbörner, 2001). In this regard, there are, however, few references demonstrating lower AFM2 toxicity in infants than that of AFM1. Following the precautionary principle, we feel entitled to consider the likely effects of the sum of these two aflatoxins. In our trial the sum of the AFM1 and AFM2 concentrations exceeded the legal limit for the content of aflatoxin M1 in baby food and infant formula (25 ng/L), as well as the legal limit for the content of AFM1 in milk (50 ng/L) (Reg. EC 1881/2006). Bianco et al. (2012) highlighted that the contemporary presence of mycotoxins AFB1, AFB2 and their respective metabolite AFM1 and AFM2 in foods, could significantly contribute to altering immune response against tumor cells, resulting in a reduction of life span and an increase of carcinogenesis.

Moreover, we found in few samples a very low excretion of unmetabolised AFB1, insufficient to estimate the rate of passage in donkey. Other authors have reported a rate of passage of AFB1 in milk (0.45% buffalo milk, Pietri et al., 2003; 0.07% sow milk, Bertuzzi et al., 2003).

Even in breast milk a remarkable concentration of AFB1 was found (Gürbay et al., 2010).

5 Conclusion

This trial showed that the presence of aflatoxins B1 and B2 in feed causes a carry-over in donkey milk. The carry-over observed on Aflatoxin M2 was higher than M1 and was 0.31% and 0.02% respectively. AFM1 carry-over was lower than that reported in other species, while AFM2 showed a

rate of passage comparable with some data from the literature. The direct transfer of AFB1 in milk was detected, although at very low amounts.

This work on the transfer of aflatoxins from contaminated feed to donkey milk is a first approach to the problem. Further studies and investigations will be useful in order to evaluate the possible effect of other variables such as the different stages of lactation and the dose of aflatoxins ingested nevertheless the aflatoxins content in donkey milk should be taken into serious consideration.

Conflict of interest Author Tozzi, author Liponi, author Meucci, author Casini, author Dall'Asta, author Intorre and author Gatta declare that they have no conflict of interest.

Statement of animal rights All applicable institutional and national guidelines for the care and use of laboratory animals were followed.

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Table 2. Average of serum parameters (n = 6) of donkeys before and after the feeding with corn naturally contaminated.

Table 3. Average of haematological parameters (n = 6) of donkeys before and after the feeding with corn naturally contaminated**.**

Figure 1. Aflatoxin M1 (AFM1) (●) and Aflatoxin M2 (AFM2) (▲) concentrations (mean±S.E.) in milk of donkeys (n = 6). A - B: steady-state period, where A was the first hour of sampling did not differ significantly (P>0.10) from the next up to B hour. E (dotted line): end time of administration of contaminated corn.