Mycotoxin Research

Influence of the ingredients used in the formulation of dairy cattle feedstuffs on aflatoxin contamination of feed and milk

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Influence of the ingredients used in the formulation of dairy cattle feedstuffs on aflatoxin contamination of feed and milk

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Abstract

Aflatoxins (AFs) are mycotoxins produced by Aspergillus species that may contaminate a wide variety of feedstuffs. Aflatoxin M$_1$ (AFM$_1$) is a hydroxylated metabolite of aflatoxin B$_1$ (AFB$_1$) and can be excreted in milk of cattle after consuming aflatoxin-contaminated feed. The aim of this research was to assess the level of AFs in dairy cattle feedstuffs (193 samples) and the level of AFM$_1$ in raw milk samples (375) estimating the AFB$_1$/AFM$_1$ carry-over from feed to milk. Moreover, the correlation between the raw materials used as ingredients of the total mixed ration (TMR) and the presence of AFs was studied. 34.7% of the feed samples analysed presented AFs in a range of 0.05-6.45 µg/kg, being 12.4% positive for AFB$_1$. 18.9% of the milk samples contained AFM$_1$ with concentrations ranging from 0.009 to 1.36 µg/kg. None of the feed samples exceeded the EU legal limit of AFB$_1$, while three raw milk samples presented levels of contamination higher than the maximum level established by the EU. A carry-over rate from 0.6 to 6% was estimated. The statistical analysis showed that maize silage, bagasse, soya bean husk, corn, alfalfa hay, cotton seed and compound feed were normally presented as ingredients of AFB$_1$-positive feed samples, thus special attention should be paid in controlling these raw materials when used in TMR preparation. Few studies have focussed before in the impact of TMR components in the AFs contamination of the final feedstuff.

Keywords

aflatoxin B$_1$, aflatoxin M$_1$, carry over, total mixed ration, raw milk

Abbreviations

AFs Aflatoxins
AFB$_1$ Aflatoxin B$_1$
AFB$_2$ Aflatoxin B$_2$
AFG$_1$ Aflatoxin G$_1$
AFG$_2$ Aflatoxin G$_2$
AFM$_1$ Aflatoxin M$_1$
UHPLC-FLD Ultra-High Performance Liquid Chromatography with Fluorescence Detector
TMR Total Mixed Ration
Introduction

Mycotoxins are secondary metabolites produced mainly by fungi belonging to the genera *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria*. In animals, the ingestion of these fungal compounds has been associated with performance problems, diseases or even death, while, in humans, the chronic intake of low levels of mycotoxins is related to different kinds of pathologies, being cancer the most important (Fink-Gremmels 1999). Fungal growth and mycotoxins production can take place in several crops and, as a result, they can reach the food and feed chain being consumed directly by humans or thorough ingestion of food of animal origin, fed with contaminated feed.

The most common mycotoxins that may occur in feedstuffs are aflatoxins (AFs), fumonisins (FBs), zearalenone (ZEN), ochratoxin A and trichothecenes (Driehuis et al. 2008). AFs are mycotoxins produced by *Aspergillus* species, mainly by *A. flavus* and *A. parasiticus*. As a consequence of fungal contamination before or after harvest, these toxins may be found in products such as nuts, dried fruits, cereals, spices, seeds, cocoa or coffee (Juan et al. 2007; Garrido et al. 2012). Up until now, more than 20 different types of AFs have been described; however, the most important in foods are aflatoxin B$_1$ (AFB$_1$), aflatoxin B$_2$ (AFB$_2$), aflatoxin G$_1$ (AFG$_1$), aflatoxin G$_2$ (AFG$_2$) and aflatoxin M$_1$ (AFM$_1$).

AFs are highly toxic, carcinogenic, mutagenic and teratogenic compounds (Bakirdere et al. 2012). AFB$_1$ is the most toxic AF and it is considered as the most powerful natural hepatocarcinogenic agent in mammals. The International Agency for Research on Cancer (IARC) has classified this toxin as a Group 1 human carcinogen (IARC 2012).

AFM$_1$ is a hydroxylated metabolite of AFB$_1$ and can be excreted in milk of cattle that have been fed with aflatoxin-contaminated feed (Fallah et al. 2009; Iqbal et al. 2015). AFM$_1$ is not as toxic as AFB$_1$ and has been classified by IARC as Group 2B as possibly carcinogenic to humans (IARC 1993). AFM$_1$ can be detected in milk of animals that have consumed feedstuff with AFB$_1$ within 24-48 h and it almost disappears after 72 h (Martins and Martins 2000; Battacone et al. 2003). The transformation of AFB$_1$ into AFM$_1$ and its presence in the secreted milk depends on several factors related with feed, metabolism, weather and geographical location of dairy farms (Masoero et al. 2007; Iqbal et al. 2013). The extent of carry-over is influenced by various nutritional and physiological factors, including regimes, rate of ingestion, health of the animal, hepatic biotransformation capacity, and milk production. Consequently, the rate of absorption of AFs and the excretion of aflatoxins varies between animal, from day to day, and also from one milking to the next. It has been reported that the predicted
rate of AFB1/AFM1 carry-over from feed to milk is approximately 0.3-6% (Heshmati and Milani 2010). Due to its high toxicity, legal limits of AFB1 in animal feed have been established by authorities. Directive 2002/32/EC sets an upper limit of 5.0 µg/kg in complete feedstuffs for dairy cattle with a moisture content of 12% (EC 2002). In addition, in order to reduce human exposure to AFM1, the European Commission Regulation 1881/2006 sets a maximum permissible limit of 0.05 µg/kg for AFM1 in raw milk, heat-treated milk and milk for the manufacture of milk-based products (EC 2006). Precisely because of the small amounts of toxin allowed, it is necessary to develop sensitive and specific analytical methods which allow the detection of very small amounts of AFs in animal feed, as well as their quantification.

Nowadays, dairy cows are usually fed using the total mixed ration (TMR) method, that consists on the supply of forages together with different kinds of concentrates or by-products (such as whole cottonseed or compound feed), grains, protein supplements, minerals and vitamins in specific quantities which are mixed thoroughly making up a balanced ration. TMR used to feed dairy cattle is usually enriched with concentrates to achieve a high milk yield. Concentrates are low-fibre, high-energy material used as components of the ration for dairy cattle in order to raise the energy level and to increase the level of proteins, minerals (macro and micro) and fat-soluble vitamins, compensating any other deficiencies of the total ration derived from the use of the forage portion. These concentrates may be an important source of mycotoxins, as well as other materials such as cereal grains or soybean products, press cakes from oil plants (Nawaz et al. 1997; Scudamore and Livesey 1998; Placinta et al. 1999), and preserved feeding stuffs like silage, hay or straw (O’Brien et al. 2005; Mansfield and Kuldau 2007).

The aims of this work were to evaluate the occurrence of AFB1, AFB2, AFG1 and AFG2 in different dairy cattle TMR samples from farms located in four Spanish areas during 2016-2018 using an in-house validated method. Moreover, the level of AFM1 in milk samples from cows fed with the mentioned feed was assessed in order to calculate the AFB1/AFM1 carry-over from feed to raw milk. The possible relation between the TMR composition and the presence of AFs in feed and, consequently, in milk, was also studied.

Materials and methods

**Sampling**

From February 2016 to January 2018, a total of 193 different feedstuff samples and 375 milk samples were collected from dairy farms located in different areas of Spain (Cantabria, Castilla-León, Cataluña and Galicia).
Farms with previous problems related to AFs, due to weak food safety management systems, were specifically selected. Each farm provided a bulk sample of 4-5 kg of TMR in order to have a representative fraction of the whole. The bulk sample was pooled from smaller samples collected through the day from a single batch while it was consumed by the cattle. Farmers were asked to provide a report containing the composition of each TMR sample and also the composition of the compound feed used as an additional TMR ingredient. The most common ingredients of the feed samples are shown in table 1. In addition, 37 raw materials used as TMR components including cotton seed (n=12), corn (n=6), compound feed (n=5), soya bean (n=2), okara (n=2), maize silage (n=2), dried mixture (n=1), barley silage (n=1), ray grass silage (n=1), sugar beet pulp silage (n=1), alfalfa silage (n=1), immature corn silage (n=1), sugar beet pulp (n=1) and soya bean husk (n=1), were received and they were analysed separately. Raw milk samples were collected in the dairy farms from the cows that had been fed with the sampled feed 24 hours and 48 hours before. All samples were stored at -18 °C until analysis.

Reagents and solutions

The analytical standards of AFB₁, AFB₂, AFG₁, AFG₂ and AFM₁ were supplied by Sigma (Sigma–Aldrich, Alcobendas, Spain). Acetonitrile and methanol were both HPLC grade and were obtained from Scharlab (Sentmenat, Spain). Acetic acid glacial was supplied by Fisher Scientific (Loughborough, UK). Filter paper (Whatman No. 113) was purchased from Whatman (Maidstone, UK). Immunoaffinity chromatography (IAC) columns for AFB₁, AFB₂, AFG₁ and AFG₂ (Easi-extract® Aflatoxin) and for AFM₁ (Aflaprep® M Wide) were purchased from R-Biopharm (Rhône LTD Glasgow, UK). Pure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Phosphate buffer saline (PBS) was prepared with potassium chloride (0.2 g) (Panreac, Castellar del Vallés, Spain), potassium dihydrogen phosphate (0.2 g) (Panreac), disodium phosphate anhydrous (1.16 g) (Panreac) and sodium chloride (8.0 g) (Panreac) in 1 L of pure water; the pH was brought to 7.4.

Preparation of stock standard solutions

Standard solutions of AFB₁, AFB₂, AFG₁ and AFG₂ were dissolved in methanol at a concentration of 250 mg/L and stored at 4 °C in a sealed vial until use. The concentrations in the stock solutions were checked by UV spectroscopy according to AOAC Official Methods of Analysis, chapter 49 (Horwitz and Latimer 2006). Working standard solutions (0.1-20 μg/L for AFB₁ and AFG₁, and 0.05-10 μg/L for AFB₂ and AFG₂) were prepared by appropriate dilution of known volumes of the stock solution with methanol:H₂O (50:50, v/v) and used to obtain calibration curves in the chromatographic system. The standard of AFM₁ was dissolved in...
acetonitrile at a concentration of 1.0 mg/L and stored at 4 °C in a sealed vial until use. Working standards (0.1, 0.25, 0.5, 1, 2, 5, 10, 25, 50 µg/L) were also prepared by appropriate dilution of known volumes of the stock solution with mobile phase and used to obtain calibration curves in the chromatographic system.

**Feed samples analysis**

Before the analysis, the samples were dried during 24 hours at 60 °C and ground into fine powder. TMR samples were extracted and cleaned up according to the Easi-extract® Aflatoxin manual slightly modified in order to obtain higher recoveries. Briefly, five grams of feed sample were extracted with 40 mL of acetonitrile: H2O solution (90:10, v/v) and kept in an ultrasonic bath for 10 min. The sample then was centrifuged for 10 min at 4,676 g. Three mL of supernatant were diluted with 72 mL of PBS and drained through the IAC column. The column was washed with 20 mL of PBS and AFs were eluted by applying 1 mL of methanol grade HPLC and 1 mL of milli-Q water, consecutively. Before the injection in the chromatographic system, the final extract was filtered through a 0.22 µm PTFE disposable syringe filter (Kinesis, Cambridgeshire, UK).

The equipment used for the detection of AFs was an Agilent 1260 Infinity Quaternary LC system (Agilent Technologies, Santa Clara, California, United States) with a quaternary pump, an auto sampler, a vacuum degasser and a fluorescence detector. The chromatographic separation was carried out in a Poroshell 120 EC-C18 UHPLC column (2.7 µm particle size, 4.6 x 50 mm; Agilent Technologies) protected with a Poroshell 120 EC-C18 UHPLC Guard 3PK (2.7 µm particle size, 4.6 x 5 mm; Agilent Technologies). In order to enhance and confirm AFB1 and AFG1 detection, a post-column derivatization with a LCTech UVE photochemical system (LCTech GmbH, Obertaufkirchen, Germany) was performed. The fluorescence detector was set at wavelengths of 365 nm and 440 nm for excitation and emission, respectively. The mobile phase consisted of acetonitrile:methanol:water (10:20:70, v/v) and the flow rate was 1.2 mL/min. The temperature of the column was set at 40 ºC, and the injection volume was 50 µL.

**Milk samples analysis**

Milk analysis was carried out with immunoaffinity columns, according to the ISO official method (ISO 2007). Milk samples were placed into 50-mL centrifuge tubes and were warmed to 37 ºC for 30 minutes. Then, they were centrifuged for 10 min at 4,534 g. The upper cream layer was removed and the defatted supernatant was filtered with No. 113 Whatman filter papers. 40 mL of this fraction were passed through the IAC Aflaprep® M Wide column. After this, the column was washed with 20 mL of PBS and, finally, eluted twice with 1.25 mL of acetonitrile:methanol (60:40, v/v). The eluent was dried under nitrogen stream and then it was resuspended with
300 µL of mobile phase consisting of acetonitrile:methanol:0.1% acetic acid (5:15:80, v/v). Before the injection in the chromatographic system, the final extract was filtered through a 0.22 µm PTFE disposable syringe filter.

The equipment used for the detection of AFM was an Agilent 1260 Infinity Quaternary LC system (Agilent Technologies) with a quaternary pump, an auto sampler, a vacuum degasser and a fluorescence detector. The chromatographic separation was carried out in a Poroshell 120 EC-C18 UHPLC column (2.7 µm particle size, 4.6 x 50 mm; Agilent Technologies) protected with a Poroshell 120 EC-C18 UHPLC Guard 3PK (2.7 µm particle size, 4.6 x 5 mm; Agilent Technologies) at a temperature of 40 °C. Excitation and emission wavelengths were set at 360 nm and 450 nm, respectively. The mobile phase consisted of acetonitrile:methanol:0.1% acetic acid (5:15:80, v/v) and the flow rate was 0.8 mL/min. The injection volume was 50 µL.

**Statistical analysis**

The analysis of the data was performed using JMP Pro 13. Firstly, ANOVA was used so as to know if there were significant effects of year, farm and composition of TMR samples on aflatoxin levels in both feed and milk samples. A multivariate partial least square regression (PLS) was carried out in order to determine which components of the feedstuffs could explain the presence of AFs in feed. VIP (Variable Importance in Projection) scores were also calculated, which are a measurement of a variable’s importance in the PLS model. They summarize the contribution of a variable to the model; higher VIP scores allow to identify the most contributory variables in class discrimination in the PLS model. Finally, correlation analysis was carried out in order to study the relationship between AFB1 presence in feed and AFM1 in milk.

**Results**

**AFs occurrence in feed and milk samples**

From February 2016 to January 2018, a total of 193 different TMR samples and 375 milk samples were analysed. 67 feed samples were contaminated with AFs and 71 milk samples were positive for the presence of AFM1. A summary of the occurrence and the range of concentrations of AFs in feed and AFM1 in milk is given in Tables 2 and 3.

In total, 24 feed samples contained AFB1, 9 were positive for AFB2, 47 for AFG1 and 13 for AFG2. Therefore, AFB1 and AFG1 were more frequently detected than AFB2 and AFG2. The concentration of AFB1 was always lower than the EU limit of 5.0 µg/kg for complete feedstuffs for dairy cattle and only one AFG1-positive sample had a toxin concentration that could be considered reasonably high (6.45 µg/kg). The number of AFB1-positive...
samples was higher in 2016, while in 2017 was AFG\textsubscript{1} the most frequent toxin found. Moreover, there seemed not to be significant differences among the four different locations under study (data not shown).

Regarding ingredients that were studied separately, AFs were not found in any of the dried mixture, maize silage, barley silage, sugar beet pulp silage, alfalfa silage, immature corn silage, sugar beet pulp, okara and soya bean husk samples. On the other hand, positive results for the presence of AFs were obtained from the analysis of corn, cotton seed, compound feed, soya bean and ray grass silage samples (Table 4).

AFM\textsubscript{1} was present in 18.4\% of raw milk samples. 19.02\% and 18.32\% of the samples obtained 24 h and 48 h after feeding, respectively, presented AFM\textsubscript{1}, therefore significant differences were not found between them.

Figure 1 shows the distribution of AFM\textsubscript{1} during the length of the study. The number of positive samples was clearly higher in 2016 than in 2017 and the season when more positive results were obtained was from May to July in 2016. Three raw milk samples exceeded the EU limit of 0.05 µg/kg.

**Relationship between TMR composition and AFs occurrence in feed and milk samples**

PLS multivariate regressions were carried out in order to assess the impact of the TMR components in AFs, AFB\textsubscript{1} and AFG\textsubscript{1} presence (Figure 2). The first model explained 49\% of variability, the second 64\% and the third one 37\%.

AFs-positive samples usually contained maize silage, barley silage, bagasse, okara, dehydrated ray grass, straw and corn. Moreover, it has to be pointed out that the compound feed was related to the presence of group B aflatoxins in feed samples (Figure 2A). AFB\textsubscript{1}-positive feed samples presented maize silage, bagasse, soya bean husk, corn, alfalfa hay, cotton seed and compound feed as ingredients (Figure 2B). As for AFG\textsubscript{1}, maize silage, alfalfa silage, barley silage, okara, soya bean husk, straw, dehydrated alfalfa and cotton seed were positively related to the presence of the toxin (Figure 2C).

Due to the weight of the compound feed in the presence of AFB\textsubscript{1} in feed samples, a PLS analysis was carried out taking into account the components of the compound feed separately, instead of considering it as a unique element. It was not possible to get from all farmers the complete information of each TMR sample (including compound feed composition), for this reason, the analysis was performed considering only those samples whose information was available (n=33). In this subset of samples, barley silage, soya bean, corn, barley and alfalfa hay were the ingredients present in AFB\textsubscript{1}-positive samples. They also contained corn, cotton seed and wheat as ingredients coming from the compound feed (Figure 3A). With this PLS model a 91\% of the variability was explained.
As far as AFM$_1$ is concerned, milk positive samples were obtained from cows that had been fed with feed that usually contained ray-grass silage, wheat silage, barley silage, bagasse, dehydrated ray-grass, straw, corn, alfalfa hay and cotton seed. Also, corn, barley and cotton seed as part of the compound feed (Figure 3B). 74% of the variability was explained by the PLS model, in this case.

**Carry-over from feed to milk**

A total of fifteen AFM$_1$-positive milk samples came from cows that had been fed with AFB$_1$-positive feed. However, eight feed samples contained AFB$_1$, but milk samples obtained from cows fed with these feed turned out to be negative for the presence of AFM$_1$. Conversely, 27 AFM$_1$-positive samples came from cows fed with feed samples that were not contaminated by AFB$_1$. As a result, the correlation coefficient found between AFB$_1$ and AFM$_1$ was 0.4774 in milk samples obtained 24 hours after having been fed with feed samples, and 0.4529 in those samples obtained after 48 hours.

Taking into account the cases in which AFs were present in both feed and milk, the daily feed intake and the milk yield by each cow, it was established that the carry-over of AFB$_1$ in feed into AFM$_1$ in milk varied from 0.6 to 6%.

**Discussion**

**Incidence of AFs in feed**

The presence of AFB$_1$ in animal feeds has been legislated in different countries in order to avoid the possible toxic effects related with its ingestion both in animals and in humans. The EU has set an upper limit of 5.0 µg/kg in complete feedstuffs for dairy cattle with a moisture content of 12% (EC 2002). However other countries are less restrictive and have established higher limits, such as USA, which have set an action level of AFs of 20.0 µg/kg in feeds and ingredients for dairy animals (FDA 1996).

The number of AF-contaminated samples found in this study was low, with only one sample containing a relatively high level of AFG$_1$ (Table 2). Our results show that 34.7% of the samples studied were contaminated by AFs in a range of 0.05-6.45 µg/kg. The most frequently found toxins were AFB$_1$ (12.4%) and AFG$_1$ (24.4%). In agreement with our research, similar results have been obtained, although it is worth noting that the farms included in our study were selected specifically due to their previous problems with AFs. Many studies found AFs in dairy feedstuffs, although only a small proportion of the contaminated samples exceeded the maximum tolerable EU limit of 5.0 µg/kg. Also in Spain, Hernández-Martínez and Navarro-Blasco, (2015) found that 90% of the dairy cow feedstuff samples under study showed detectable concentrations of AFs, without exceeding the
legal limit. In China, Han et al. (2013) obtained 53.5% AFs-positive feed samples, being 17.5% positive for AFB$_1$ with concentrations from 0.05 to 3.53 µg/kg. Decastelli et al. (2007) in Italy and Sassahara et al. (2005) in Brazil, detected also a low number of contaminated samples, 8.1% and 17%, respectively. In Turkey, Sahin et al. (2016) found that 26.3% of the dairy cattle feedstuffs contained AF and the concentrations ranged from 0.278 to 8.43 µg/kg, although only two samples presented levels higher than 5.0 µg/kg.

By contrast, other studies found a relatively low number of contaminated feed samples but some of them presented high values of AFs, widely exceeding the established legal limit. In Portugal, Martins et al. (2007) carried out a 10-year study finding 37.4% positive samples with a level of contamination ranging from 1 to 15 µg/kg, showed levels of AFB$_1$ above the EU limit (from 5.1 to 74 µg/kg). Similarly, Pleadin et al. (2015) obtained 22.2% AFB$_1$-positive samples and 12.3% had concentrations over the limit, showing, occasionally, very high values being the maximum found 304.6 µg/kg.

Conversely, other studies have found a higher number of samples containing AFs and in higher concentrations in dairy cattle feed. A research carried out in Turkey (Kocasari et al. 2012), observed that AFs were presented in 61.7% of samples in concentrations ranging from 3.82 to 54.34 µg/kg. In Iran, Ehsani et al. (2016) obtained 41.9% of contaminated samples with concentrations between 1.87 and 19.41 µg/kg and 42% contained AFB$_1$ at the level of 10-15 µg/kg. All the samples examined by Gizachew et al. (2016) in Ethiopia presented AFB$_1$ and 26.2% exceeded such a high concentration as 100 µg/kg. Recently, Ismail et al. (Pakistan, 2017) and Mohammed et al. (Tanzania, 2016) found 30.5% and 61.5% of the samples, respectively, with concentration of AFB$_1$ over the EU permissible limit.

**AFM$_1$ in milk**

Although the problem of AFM$_1$ in milk has been known for a long time, recently several cases of milk contaminated with this toxin in Europe have revealed the extent of the problem, both from the point of view of Public Health and from an economic angle. Thus, for example, in a sporadic outbreak that took place in the South of Spain, two million litres of raw milk, prior to its treatment and commercial sale, had to be destroyed in 2013 due to its contamination by AFM$_1$ above the levels legally allowed in the EU, with a cost of more than 760,000 euros (El Mundo, 2013). It cannot be ruled out that the emergence of outbreaks of contamination by AFM$_1$ in milk could be due, among other possible causes, to climate change, which may be causing a change in the pattern of distribution of Aspergillus aflatoxigenic strains in Europe and favouring their growth.
As well as with AFB1 in animal feed, there is current regulation for the maximum allowable concentration of AFM1 in milk in order to reduce the exposure to this toxic metabolite. The EU has set a maximum of 0.05 µg/kg while in the USA it is allowed to reach levels of AFM1 up to 0.5 µg/kg (FDA 1996). These standards have been adopted by numerous countries (FAO 2004; EC 2006).

The present study analysed raw milk samples obtained after 24 and 48 hours, respectively, from cows that had been fed with TMR samples. 71 out of 375 milk samples (18.9%) contained AFM1 in concentrations above the limit of detection in a range from 0.009 to 1.36 µg/kg, with only three samples exceeding the EU limit. These results are in line with data obtained by Han et al. (2013) who observed that 22.5% of raw milk samples from China contained AFM1 ranging from 0.005 to 0.06 µg/kg. Seven samples contained levels of toxin over the EU legal limit. Sahin et al. (2016) in Turkey, found out 21.1% of contaminated raw milk samples in a range of 0.011-0.1 µg/kg, containing three samples concentrations of AFM1 that exceeded the EU limit. Golge (2014), also in Turkey, detected AFM1 in 30.1% of the raw milk samples analysed at concentrations between 0.025 and 1.01 µg/kg. Camaj et al. (2018), in Kosovo, detected AFM1 in 38% of milk samples analysed. From 2013 to 2015, Zheng et al. (2017), in China, found out a mean of 21.2% of contaminated samples. However, in Italy, several authors (Decastelli et al. 2007; Armorini et al. 2016; De Roma et al. 2017) reported very low levels of contamination in cow milk and usually no sample exceeded the EU regulation limit.

By contrast, a wide number of authors detected higher percentages of contaminated samples with concentrations that frequently exceeded the legal values. Bakirci (2001) in Turkey, found AFM1 in 87.8% of the samples examined and with 44.3% of the cases surpassing the EU limit. In Ethiopia, Gizachew et al. (2016) reported that all the milk samples analysed were positive for the presence of AFM1 and only 8.2% contained less than or equal to 0.05 µg/kg of AFM1. Mohammed et al. (2016) in Tanzania detected that 83.8% of the milk samples were contaminated with AFM1, exceeding all of them the EU established limit. These differences could be attributed to several factors including different analytical techniques, sample size and composition, season of the year, livestock management and dairy processing systems.

**Carry-over of AFB1 from feed to AFM1 in milk**

AFB1 is transformed to AFM1 in milk when lactating animals are fed with contaminated feed. However, only a small proportion of AFB1 in feed leads to AFM1 in the milk of dairy cows (Pittet 1998). Consequently, the incidence of AFB1 in feed is much higher than the incidence of AFM1 in raw milk.
Our study gave as a result that only fifteen raw milk samples positive for the presence of AFM$_1$ were obtained from cows that had been fed with contaminated feed having, consequently, a moderate positive correlation between AFM$_1$ contamination in milk and the level of AFB$_1$ contamination in feed. The carry-over rate varied from 0.6 to 6%, in agreement with data reported in other studies (Veldman et al. 1992; Pittet 1998; Unusan 2006; Iqbal et al. 2015). Other authors found lower rates of transformation that ranged from 1 to 3% (Diaz et al. 2004; van Eijkeren et al. 2006; Masoero et al. 2007). Sumantri et al. (2012) established that the carry-over was only 0.1%.

Despite all feed samples presented concentrations of AFB$_1$ below the EU limit, three raw milk samples exceeded the legal value for AFM$_1$. Moreover, 27 milk samples were positive for AFM$_1$ while correspondent feed samples were not contaminated with detectable levels of AFB$_1$. Conversely, the presence of AFB$_1$ in feed samples was not always reflected in milk. This fact has also been previously reported by other authors (Battacone et al., 2003; Gizachew et al., 2016; Han et al., 2013). Therefore, analysed levels of AFB$_1$ in feed within the limits do not guarantee a content of AFM$_1$ in milk under the permitted concentrations. A possible explanation could be the heterogeneous distribution of mycotoxins in raw materials and the problems derived from the sampling procedure. Only some sections of the feedstuffs are likely to be highly contaminated whereas most of the feed will probably not present any mycotoxin (Miraglia et al. 2005). Consequently, the amount of toxin could be underestimated if the contaminated fractions are not analysed. In a like manner, if only the heavily AF-

TMR composition

Concentrates used in order to enrich feed for dairy cattle can represent up to a 70% of the daily feed ration, in our case they accounted for up to 40%. As a consequence, this ingredient may be an important source of mycotoxins due to its possible contamination with AFs. These toxins can be also found in cereal grains or
soybean products as well as in by-products from oil plants such as peanuts, sunflower seed, cotton seeds, palm kernels and copra (Nawaz et al. 1997; Scudamore and Livesey 1998; Placinta et al. 1999). Another source of AFs in the diet of dairy cows comes from the consumption of preserved feeding stuffs like silage, hay or straw (O’Brien et al. 2005; Mansfield and Kuldau 2007). After a long storage period, silage can be spoiled by some fungal species, being Aspergillus species (A. fumigatus and A. flavus) some of them (Cole et al. 1977). However, AFs only have been sporadically detected at low levels in forages. Maize has been traditionally related with the presence of AFs in dairy cattle diet, too (Whitlow and Hagler Jr. 2005), and it is usually one of the main components of animal feed (EFSA, 2013; Battilani et al., 2012).

The multivariate PLS regression carried out led us to identify which components of the total ration could have influenced on the presence of AFs in the feed (Figure 2 and Figure 3). Table 4 shows those raw materials that were positive for the presence of AFs and the range of concentrations. Positive samples of corn and compound feed could confirm the results of the PLS analysis in which it was observed that these ingredients seemed to be related with the presence of AFB₁ in the feed samples (Figure 2B and Figure 3A). The highest concentration of AFB₁ (2.44 µg/kg) was found in one of the compound feeds which was also positive for AFB₂, AFG₁ (3.25 µg/kg) and AFG₂. In this case, it was also analysed the correspondent TMR and it was found out to be positive for the presence of AFB₁, AFB₂ and AFG₁. In addition, milk samples from cows fed with this feed were contaminated with AFM₁. Some samples of cotton seed and corn were also positive for the presence of AFs. However, although cotton seed presented sometimes high concentrations of AFs, the toxins did not appear in the correspondent TMR sample. It may be explained because compound feed used to represent a relatively high proportion of the whole whereas the cotton seed represented normally a small portion (0.68-5.51%) of the total ration (Table 1).

Our positive outcomes in cotton seed and in corn are in agreement with other studies related to the analysis of feed ingredients. Liu et al. (2016) found that cottonseed was the most seriously contaminated feed ingredient examined. They also found that a high percentage of corn samples presented AFB₁. Sadegh et al. (2013) in Iran found out that cotton seed and sunflower meal samples were the most contaminated materials with AFB₁. The toxin was also presented in alfalfa, straw, rapeseed, cotton seed, corn silage and soybean meal samples although the highest concentration was in cotton seed. For Pleadin et al. (2015), maize was the most contaminated component although they also had positive AFB₁-samples of wheat, barley and oat. Whole cottonseed, as well as other oilseed such as soybean, flaxseed and sunflower, could be used as fat sources in dairy cattle feed. These ingredients allow a slow release of lipids in the rumen. Whole cottonseed has 22% crude protein, 20% ether...
extract and 83% of total digestive nutrients, being, therefore, an excellent source of supplemental fat to increase
diet energy density. Moreover, it seemed to be highly effective in maintaining milk fat. Despite all of these
advantages, it could be related to the presence of AFs, therefore, it should be studied if it would be possible the
replacement of these ingredient with others with similar nutritional properties (De Almeida et al. 2017).
Therefore, the level of contamination, both, in feed and milk was relatively low, in agreement with other studies
carried out recently and this is probably related to the increasing surveillance measures and quality control of
raw materials used for manufacturing feeds as well as the application of Good Agricultural and Storage
Practices. However, in recent years, there are still outbreaks of AFM1 in milk over the legal limit and this may
be linked to the raw materials used in the formulation of feedstuffs, hence it is important to have present
information about the contribution of the ingredients on the AFB1 content of the total mixed ration (TMR) given
to cattle. Moreover, it is important to remember that, besides AFs, there are other mycotoxins that can occur in
dairy feedstuffs and feed ingredients, including deoxynivalenol, zearalenone and fumonisins, that may be also
harmful to animals and humans (Driehuis et al. 2008). Thus, mycotoxin contamination in feed and in milk
should be regularly monitored, in particular the most susceptible ingredients, in order to reduce their incidence
and their effects.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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References


of Turkey. Food Control 12:47–51. doi: 10.1016/S0956-7135(00)00020-7

species: Their health effects and determination methods in different foodstuffs. Cent Eur J Chem 10:675–


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International Agency for Research on Cancer (IARC) (1993) Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. Lyon (France)


Kocasari FS, Tasci F, Mor F (2012) Survey of aflatoxin M1 in milk and dairy products consumed in Burdur,


**Figure captions**

**Fig. 1** AFM$_1$-positive milk samples from February 2016 to January 2018

**Fig. 2** Importance of the TMR components (VIP) on the presence of AFs (A), of AFB$_1$ (B) and of AFG$_1$ (C) in feed samples

**Fig. 3** Influence of the TMR components (VIP) on AFB$_1$ presence in feed (A) and on AFM$_1$ in milk from cows fed with feed (B) taking into account the ingredients of the compound feed

**Tables**

**Table 1.** Main ingredients of total mixed ration samples, number of samples that contained each of them and percentage of inclusion in the feed.

<table>
<thead>
<tr>
<th>TMR component</th>
<th>No. of samples (% total)</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound feed</td>
<td>145 (75.13%)</td>
<td>1.61-59.18</td>
</tr>
<tr>
<td>Maize silage</td>
<td>107 (55.44%)</td>
<td>9.92-76.92</td>
</tr>
<tr>
<td>Straw</td>
<td>95 (49.22%)</td>
<td>0.68-10.71</td>
</tr>
<tr>
<td>Dehydrated alfalfa</td>
<td>78 (40.41%)</td>
<td>2.28-25.66</td>
</tr>
<tr>
<td>Alfalfa hay</td>
<td>63 (32.64%)</td>
<td>1.63-32.65</td>
</tr>
<tr>
<td>Corn</td>
<td>51 (26.42%)</td>
<td>0.11-22.83</td>
</tr>
<tr>
<td>Grass silage</td>
<td>47 (24.35%)</td>
<td>9.89-89.29</td>
</tr>
<tr>
<td>Bagasse</td>
<td>40 (20.73%)</td>
<td>3.56-25.81</td>
</tr>
<tr>
<td>Immature corn silage</td>
<td>33 (17.10%)</td>
<td>3.76-19.05</td>
</tr>
<tr>
<td>Sugar beet pulp</td>
<td>30 (15.54%)</td>
<td>0.93-22.79</td>
</tr>
<tr>
<td>Cotton seed</td>
<td>30 (15.54%)</td>
<td>0.68-5.51</td>
</tr>
<tr>
<td>Ray grass silage</td>
<td>28 (14.51%)</td>
<td>7.60-75.95</td>
</tr>
<tr>
<td>Alfalfa silage</td>
<td>21 (10.88%)</td>
<td>5.71-44.78</td>
</tr>
<tr>
<td>Soya bean</td>
<td>21 (10.88%)</td>
<td>3.04-14.47</td>
</tr>
<tr>
<td>Barley</td>
<td>21 (10.88%)</td>
<td>1.13-8.10</td>
</tr>
<tr>
<td>Raw material</td>
<td>No. of total samples</td>
<td>No. of positive samples (range, µg/kg)</td>
</tr>
<tr>
<td>----------------------</td>
<td>----------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>Ray grass silage</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Corn</td>
<td>6</td>
<td>3 (0.24-1.05)</td>
</tr>
<tr>
<td>Cotton seed</td>
<td>12</td>
<td>5 (0.08-1.97)</td>
</tr>
<tr>
<td>Soya bean</td>
<td>2</td>
<td>1 (0.26)</td>
</tr>
<tr>
<td>Compound feed</td>
<td>5</td>
<td>2 (0.17-2.44)</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 3