Aflatoxin M₁ and Ochratoxin A in Raw Bulk Milk from French Dairy Herds

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ABSTRACT

Mycotoxins in milk are a public health concern and have to be regularly monitored. A survey on the presence of aflatoxin M₁ (AFM₁) and ochratoxin A (OTA) in raw bulk milk was conducted in 2003 in the northwest of France, the main French milk-producing basin. Randomly selected farms (n = 132) were characterized by a diet based on corn silage and containing a large proportion of on-farm produced cereals, feeding sources that are frequently contaminated by mycotoxins. Farms were surveyed twice in winter and in summer. At each sampling time, a trained surveyor completed a questionnaire recording farm management procedures and production traits. The AFM₁ was found in 3 out of 264 samples but at levels (26 ng/L or less) that are below the European legislation limit of 50 ng/L. Traces of AFM₁ (less than 8 ng/L) were also found in 6 other samples. The OTA was detected in 3 samples also at low levels, 5 to 8 ng/L. Farms that tested positive to the presence of mycotoxins, 12 in total including 6 farms that had traces of AFM₁, differed from negative farms by a more extensive use of total mixed rations, 58 vs. 27%. In addition, the positive farms tended to have lower milk yields. Although the incidence of milk contamination with AFM₁ and OTA at the farm level was low during the period studied, production and management data from the surveyed farms suggest a link between feeding management practices and mycotoxin contamination.

Key words: mycotoxin, milk safety, French dairy herd, feeding management

INTRODUCTION

Aflatoxins and ochratoxins are highly toxic secondary metabolites produced by several *Aspergillus* and *Penicillium* species that can be found in cow's milk. Afla-

toxin M₁ (AFM₁), the major metabolite of aflatoxin B₁ (AFB₁), and ochratoxin A (OTA) are classified by the International Agency of Research on Cancer as class 2B, possible human carcinogens (IARC, 1993). Aflatoxin M₁ level in milk and milk products is regulated in several countries; the European Union limit of 0.05 µg/kg being one of the lowest in the world (EFSA, 2004). In contrast, no regulation for OTA in milk exists, even though it has been suggested that the level of this toxin in cow's milk may exceed the tolerable daily intake of 5 ng/kg of BW per day for small children in some areas in Norway (Skaug, 1999).

The presence of mycotoxins in dairy products reflects the contamination of feedstuffs. Following ingestion of contaminated feeds, OTA is largely transformed by rumen microorganisms into the less toxic metabolite ochratoxin α (OTα; Kiessling et al., 1984). Ochratoxin A and OTα are mainly eliminated in the urine and feces, but they can also be found in milk. In contrast, AFB₁ is poorly degraded by rumen microorganisms (Kiessling et al., 1984). Absorbed AFB₁ is principally metabolized in the liver into AFM₁, a metabolite as toxic as the parent toxin, which appears in milk. The amount of AFM₁ found in milk represents normally 1 to 2% of the ingested AFB₁. However, it can be as high as 6% in high-producing cows (Veldman et al., 1992). For OTA, no information is available on the rate of transfer of this toxin into milk for dairy cows. In dairy sheep, the carryover is less than 1% (Boudra et al., 2005). Among the various feedstuffs susceptible to mycotoxin contamination, cereals and cereal by-products are the major source of AFB₁ and OTA. Aflatoxins are predominantly produced in hot climates. In temperate countries, the strict control of imported feeds has limited the cases of milk contamination above the maximum tolerable limits. However, the presence of aflatoxin was recently reported in feeds grown in Europe (EFSA, 2004). The emergence of aflatoxin in the European continent is concurrent with the increase in the annual average temperature registered in the past decade.

The presence of mycotoxins, aflatoxins in particular, is tightly controlled by feed manufactures and, conse-

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quently, contamination in commercial feeds is rare. A major risk of contamination, however, arises from cereals and forages, such as corn silage (Scudamore and Livesey, 1998; Garon et al., 2006), that are produced on the farm and are exposed to adverse climatic conditions or improperly conserved.

Investigations on milk AFM₁ contamination are regularly conducted in EEC countries (EFSA, 2004), but there is little information on the contamination of milk by other mycotoxins. Ochratoxin A is frequently found in Europe in feeds and foods of plant origin (Pittet, 1998; Scudamore and Livesey, 1998; Araguas et al., 2005). Occurrence of OTA in animal products has also been reported, particularly in meat and offal from pigs (Dragacci et al., 1999; Matrella et al., 2006). However, there is scarce information on the presence of OTA in cow's milk (Breitholtz-Emanuelsson et al., 1993; Valenta and Goll, 1996; Skaug, 1999), and no data are available in France concerning milk contamination by this mycotoxin.

The present study examined the presence of AFM₁ and OTA in bovine milk collected from French dairy herds receiving a diet of on-farm-produced cereals and corn silage, a feeding system that maximizes the risk of mycotoxin exposure.

MATERIALS AND METHODS

Farm Selection

The dairy farms studied were in 4 regions (Aquitaine, *n* = 34; Brittany, *n* = 33; Poitou-Charentes, *n* = 32; and Pays de Loire, *n* = 33) of western France, the main area in France for both corn and milk production (Sigwald and Dervishi, 2005). Farms were randomly selected from the national DHIA database according to the following criteria: Holstein breed, more than 20 cows, and corn silage constituting at least 50% of the dietary DM. The study did not involve any payment to the selected farmers, who were considered good managers and were advised regularly by competent technicians.

Data and Sample Collection

The DHIA technicians conducted 2 questionnaire surveys (February to March 2003 and September to October 2003) on the 132 selected farms. For each survey, the technicians collected data concerning farm characteristics, feeding practices, milk production, presence of molds in forages, and health problems (suspected or confirmed) due to mycotoxins. Moreover, the DHIA surveyors gave insight into the quality of the feeding management, and they collected raw bulk milk samples (*n* = 264). Milk samples were preserved by adding 1.5 µg/mL of NaN₃, kept on ice during transport

from the farm to the laboratory, and stored at -20°C until analysis.

Mycotoxin Analysis

The mycotoxins AFM₁ and OTA were analyzed using the official French method (NF EN ISO 1451; Dragacci and Grosso, 2001) and the method of Boudra and Morgavi (2006), respectively. Briefly, for AFM₁, 50 mL of filtered milk was applied to an immunoaffinity column (IAC, Aflaprep, R-Biopharm, Lyon, France). The IAC was then washed with 20 mL of water, and AFM₁ was eluted with 4 mL of CH₃CN. For OTA, 10 mL of acidified (pH < 2) milk samples were extracted with 10 mL of CHCl₃. The toxin in the organic layer was back-extracted with 8 mL of PBS, pH 7.6, and the top aqueous layer was then loaded into an IAC (Ochraprep, R-Biopharm). Ochratoxins were slowly eluted with 3 mL of CH₃OH.

The purified extracts of AFM₁ and OTA were evaporated to dryness at 45°C under a stream of N gas, and the dried residues were redissolved in 200 µL of mobile phase by incubation in an ultrasonic bath for 3 min. A 50-µL volume of these extract solutions was injected into a HPLC system (Thermo Finnigan, Paris, France) equipped with an automatic sampler (Spectra-Physics, Paris, France) and a fluorescence detector (FL-3000, Thermo Finnigan). Separation was performed at room temperature on a Nucleodur C₁₈ gravity column (125 × 4.6 mm, 5 µm, Macherey Nagel, Lyon, France), using an isocratic mobile phase pumped at a flow rate of 1 mL/min. The mobile phase was CH₃CN:distilled water (25 to 75 ratio) and CH₃CN:10% acetic acid (54 to 46 ratio) for AFM₁ and OTA, respectively. For fluorescence detection, excitation and emission were respectively set at 365 and 435 nm for AFM₁ and at 274 and 440 nm for OTA. Recovery and linearity were tested for both mycotoxins. In addition, the OTA analytical method was fully validated in terms of precision, accuracy, sensitivity, and stability as described previously (Boudra and Morgavi, 2006).

The calibration curve was determined daily, using a series of standard solutions in mobile phase containing different levels of each mycotoxin. The concentration of OTA and AFM₁ was calculated by using the following formula: $concentration\ (ng/mL) = \frac{M \times Vd}{V \times Vi}$, where *M* = the mass (ng) injected into the HPLC system; *V* = the volume of milk (mL) taken for analysis; *Vd* = the volume (mL) used to dissolve the dried extract; and *Vi* = the volume (mL) injected into the HPLC system. The limit of quantitation was calculated by using a signal-to-noise ratio of 3:1, and it was defined as the lowest con-

Table 1. Aflatoxin M₁ (AFM₁) and ochratoxin A (OTA) concentrations in raw bulk milk collected from French dairy farms in winter and summer 2003

Region	Number of samples	Number of positive samples (mycotoxin concentration)	
		OTA (ng/L)	AFM ₁ (ng/L)
Brittany			
Winter	33	1 (6.6)	1 (trace ¹)
Summer	33		
Pays de la Loire			
Winter	33		3 (trace)
Summer	33	1 (5.0)	
Aquitaine			
Winter	34	1 (6.4)	
Summer	34		3 (trace, 9, and 26)
Poitou-Charentes			
Winter	32		1 (trace)
Summer	32		1 (8)
All	264	3	9

¹Trace = trace concentrations that ranged from 5 ng/L to less than 8 ng/L (limit of quantification).

centration measured with satisfactory accuracy and precision.

Statistical Analysis

All data obtained from the questionnaire were checked for unlikely values and extracted from an Access 2000 relational database (Microsoft, Redmond, WA). Complete data were obtained for the whole set of farms studied. Statistical analysis was conducted using SAS/STAT 8.1 (SAS Institute Inc., Cary, NC). Statistical significance was defined at $P < 0.05$. Production and management variables were compared between herds with and without detectable AFM₁ or OTA in milk using χ^2 and Student's *t*-tests.

RESULTS

Occurrence of Mycotoxins in Raw Milk

The validation criteria obtained with quality control samples were satisfactory for both analytical methods and showed good linearity ($r > 0.999$). The limit of quantification was 8 and 5 ng/L with a mean recovery of 89.4 and 89.8% for AFM₁ and OTA, respectively.

The presence of mycotoxins in milk samples is shown in Table 1. From the 264 samples that were analyzed from the 132 farms surveyed in winter and summer in 2003, three samples (1.1%) contained AFM₁ at low levels (8 to 26 ng/L), whereas 6 samples (2.2%) contained traces (5 to less than 8 ng/L). The OTA was detected in 3 samples (1.1%), albeit at low levels (5.0 to 6.6 ng/L), whereas its metabolite, OT α , was not detected in any sample. None of these positive samples contained

both toxins, and all originated from different farms. Moreover, no effect of region or sampling period on the presence of mycotoxin in milk was highlighted.

Mycotoxins and Herd Management

Table 2 displays farm characteristics and management practices in herds that tested positive (MYCO group) or negative (ZERO group) to the presence of mycotoxins in the milk tank. Corn silage, as the major dietary source in the surveyed herds, was consumed almost yearlong in the MYCO (12 ± 0 mo) and ZERO (11.6 ± 0.9 mo) groups. The cows also notably consumed on-farm-grown cereals. Moreover, a trench silo including a conservative agent was the dominant way to store corn silage in the sampled herds. According to DHIA technicians, a great majority of farmers adequately managed the herd feeding system. A very few number of mycotoxin-induced health problems were suspected in the last 5 yr; curiously, these cases were reported in herds that tested negative.

Total mixed rations were more frequently ($P < 0.05$) used in MYCO (58.3%) than in ZERO herds (27%). Except for this feeding practice, no other management or production characteristic differed between the 2 groups of herds. Nevertheless, mycotoxin-negative farms tended ($P < 0.1$) to have a higher mean milk yield per cow (7,982 vs. 7,396 kg/yr).

DISCUSSION

The incidence of AFM₁ and OTA contamination of milk was lower in the present study than reported by recent European surveys (Breitholtz-Emanuelsson et al., 1993; Valenta and Goll, 1996; Galvano et al., 1998; Skaug, 1999; Martins and Martins, 2000; Roussi et al., 2001; Rodriguez Velasco et al., 2003). Our study confirmed the low incidence of AFM₁ in milk produced in France, as shown in a previous study (Dragacci and Fremy, 1993). The wide variations in mycotoxin levels among studies could be related to geographic and climatic differences but also to differences in feeding systems, farm management practices, and analytical methods. Contamination of feeding sources with AFB₁ varies with location, because it is influenced by weather conditions during harvest and feed storage practice. Kim et al. (2000) attributed the high incidence of AFM₁ contamination in Korea to the extensive use of cereals and the lack of pastures in dairy cattle production, whereas Saitanu (1997) attributed the high contamination observed in Thailand to the tropical climate of the country, in which temperature and humidity are optimum for mold growth and aflatoxin production. In this survey, cereals and corn silage given to cows were pro-

Table 2. Production characteristics and management practices in French dairy herds with (MYCO) and without (ZERO) detectable aflatoxin M₁ or ochratoxin A in raw bulk milk

Variable	ZERO herds (n = 120)	MYCO herds (n = 12)
Farm description		
Farm area, ha	90.6 (59.7) ¹	83.1 (46.5)
Corn area, ha	21.6 (12.0)	24.1 (18.8)
Number of cows	48.7 (19.7)	50.1 (16.7)
Milk yield, kg/cow	7,982 (1,481)	7,396 (971) [†]
Milk fat content, g/L	41.7 (2.7)	41.7 (2.5)
Milk protein content, g/L	32.3 (1.5)	32.3 (1.0)
Feed source, mo/yr		
Grazed grass	4.4 (3.5)	3.7 (3.4)
Grass silage	3.6 (4.4)	4.6 (4.2)
Corn silage	11.6 (0.9)	12.0 (0.0)
Other silage	0.3 (1.3)	0 (0)
Hay	8.5 (5.0)	8.4 (5.5)
Other forage	2.2 (4.3)	0.4 (1.2)
Soybean meal	5.7 (5.8)	6.2 (6.0)
Farm cereal concentrate	5.2 (5.8)	4.5 (6.0)
Commercial cereal concentrate	0.9 (3.0)	2.2 (4.8)
Feeding management		
Corn silage, % DM	34.6 (10.2)	34.3 (4.5)
Corn silage daily quantity, kg of DM/cow	12.6 (2.2)	13.1 (1.9)
Corn silage in trench silos, % of herds	77.9	66.7
Conservation agent in corn silo, % of herds	97.5	83.3
TMR, % of herds	25.0	58.3*
Feed management quality according to DHIA technicians, % of herds		
Very good	41.8	33.3
Good	48.4	66.7
Medium	9.2	0
Molds/mycotoxin, % of herds		
Forage with at least 1 visible mold spot	44.3	58.3
Forages with many visible molds	1.7	0
Herdsman very watchful to moldy forages	58.3	41.7
Suspected mycotoxin-induced disease: last 5 yr	2.5	0

¹Mean (SD).[†]*P* < 0.10; **P* < 0.05.

duced in 2002, an average year in terms of climate for the regions studied. However, it is worth noting that during the summer of 2003, a record heat wave was experienced in Europe. Extreme weather conditions have been associated with aflatoxin presence on corn in the Po valley of Italy (EFSA, 2004). The projected increased in global average temperature, as well as the frequency and duration of heat waves (European Environment Agency, 2005), may increase the risk of aflatoxin contamination in milk in Europe and other temperate regions soon.

The low level of OTA contamination we observed does not pose any particular risk for milk consumers. Ochratoxin A is largely metabolized in the rumen into the less toxic metabolite OT α (Kiessling et al., 1984), a metabolite that is also excreted in milk and could be used as a marker of exposure. However, OT α was not detected in any of the analyzed samples, probably because of the low binding of this metabolite to the IAC used (Boudra and Morgavi, 2006). In the absence of

a reliable immunoaffinity binder for OT α , it was not possible to attribute the observed low level of contamination to the efficiency of rumen biodegradation or just to the low level of toxin in feeds consumed by the animals.

This survey gives a snapshot of milk contamination at this particular time but does not preclude mycotoxin absence (or presence) in the future. In this study, milk samples were taken from farms in the major production basin in France. These farms were selected because the diets given to dairy cows, based on corn silage and farm-grown cereals, had the maximum risk of mycotoxin contamination. Mycotoxins in milk are indicators of feed contamination (e.g., AFM₁ is a marker for AFB₁ in feeds that appear in milk within 12 h postingestion; Frobish et al., 1986). The low incidence of milk contamination found in this study indicates, consequently, a low contamination of the ration. However, production and management data from the surveyed farms would also suggest a link between feeding management and the likeli-

hood of having a mycotoxin problem. The precise role that use of TMR may play on the prevalence of AFM₁ and OTA cannot be discerned from this work. Further experimental and observational studies must be performed to understand the underlying factors involved in the relationship between TMR and mycotoxin contamination. Moreover, the tendency for higher milk yields in the ZERO group could underline that these farmers have more accurate feeding practices than the farmers of the MYCO group.

This study showed that AFM₁ and OTA contamination of raw farm milk from French dairy farms was low during 2003, and, when detected, mycotoxin levels did not exceed legal limits. Additionally, the study emphasized the role of management practices in the prevention of mycotoxin prevalence in dairy farms.

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