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Risk factors associated with the presence of aflatoxin M1 in raw bulk milk from Argentina

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ABSTRACT

The objectives of this study were to assess aflatoxin M_1 (AFM1) contamination in bulk tank milk, and to further identify the risk factors associated with the presence of AFM1 in raw milk in Argentina. The presence of AFM1 was investigated in 160 bulk tank milk samples collected from farms located in the most important milk production region in Argentina during one year (four seasons). Samples were analysed using immunoaffinity column (IAC) cleanup and UHPLC-MS/MS method for determining AFM1 at low levels of concentrations (LOQ = 0.003 μg L^{-1}). A survey about the potential factors associated with the presence of AFM1 in milk was performed directly in the field through a questionnaire applied to the farmers. Chi-square and logistic regression were performed with presence of AFM1 in milk as dependent variable, and potential risk factors as independent variables. Incidence of AFM1 in raw milk was 38.8% and, in all samples, AFM1 levels were lower than the Southern Common Market (MERCOSUR) Regulation (maximum level accepted = 0.5 μg L^{-1}). Commercial feed consumption (OR = 4.630, $P = 0.001$), soybean expeller consumption (>0.95 kgDM/cow) (OR = 3.542, $P = 0.019$), and cotton seed consumption (>1.5 kgDM/cow) (OR = 2.949, $P = 0.089$) were associated with the incidence of AFM1 in raw milk. Despite the incidence and the level of AFM1 in milk produced and commercialized in Argentina is not a serious problem for public health. The farm breeding intensification and the supplementation with commercial feed, soybean expeller, and cotton seed seems to be the risk factors that impacts on the AFM1 milk contamination. Therefore, Argentina should improve its monitoring program on mycotoxins in animal feed and milk and improve the management practices in farms.

1. Introduction

Aflatoxins are secondary metabolites produced by specific filamentous fungi that are common contaminants of agricultural commodities (Binder, 2007). Aflatoxin M_1 (AFM1) is the hydroxylated metabolite of aflatoxin B_1 (AFB1) and is found in milk as the direct result of the intake of contaminated feeds (Creppy, 2002; López, Ramos, Ramadan, & Bulacio, 2003; Prandini et al., 2009).

AFM1 can cause DNA damage, chromosomal abnormalities, gene mutation, and cell transformation depending on the level of exposure (Van Egmond, 1989). However, it is less mutagenic and genotoxic than AFB1 (Black, McVey, & Oehme, 1992; Prandini et al., 2009). The presence of AFM1 in milk and milk products is a particular risk for humans as consequence of their negative effects in foodstuff for adults and especially children (Prandini et al., 2009).

The presence of AFM1 in milk depends on the presence of AFB1 in the feed which is influenced (among other factors) by the environmental conditions that favor mould growth and toxin production (Van Egmond, 1989). MERCOSUR establishes a legal limit of 20 μg/kg for total aflatoxins (B1, B2, G1 and G2) in maize, peanut and products from both crops destined to human consumption

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(MERCOSUR/GMC/RES N°25/02). A quantitative risk assessment conducted in Argentina (Signorini et al., 2012) showed that the principal sources of AFM1 in raw bulk tank milk were corn silage, cotton seed, and concentrated feeds. This stochastic simulation (Signorini et al., 2012) also showed that 0.81% of the bulk tank milk would exceed the AFM1 maximum level accepted by the MERCOSUR regulations (0.5 μg L^{-1}).

In Argentina there are a few studies which estimated the prevalence of AFM1 in raw milk. Alonso et al. (2010) reported an AFM1 prevalence of 64% on 94 milk samples from 47 dairy farms samples with a mean level of 0.028 μg L^{-1} (SD = 0.015 μg L^{-1}), and López et al. (2003) showed an AFM1 prevalence of 23% on 77 milk samples (mean level = 0.016 μg L^{-1}; SD = 0.007 μg L^{-1}) from diverse origin (bulk tank, commercial fluid and powdered milk). However, in both studies, exceedances of the maximum acceptable limit of AFM1 established by the MERCOSUR regulations, were not verified. Other AFM1 occurrence studies in the South American region (Brazil) were reported. Scaglioni, Becker-Algeri, Drunkler, and Badiale-Furlong (2014) analysed 40 milk samples from diverse origin (raw, pasteurized, UHT-treated and powdered milk) and reported that AFM1 was present in 29% of raw milk samples (average level = 0.835 μg L^{-1}) and all the samples showed levels that were above the legislated limit in Brazil (0.5 μg L^{-1}). In an extreme case, Picinin et al. (2013) reported 100% AFM1 occurrence in 129 raw milk samples from three climate periods, although the concentrations were below the permitted limit according to the Brazilian legislation (contamination levels ranged from 0.0002 to 0.1057 μg L^{-1}). This reflects the importance that occurrence of AFM1 in milks from different regions on this part of the continent has, and the potential hazard for consumers involved, together with eventual economic loss when international limits are exceeded.

Despite the importance that the presence of AFM1 in daily milk has for Public Health, the risk factors associated with this presence in bulk tank milk in Argentina have yet to be fully understood. The objectives of this study were to (i) experimentally assess AFM1 contamination in bulk tank milk, and (ii) to identify the risk factors in milk farms associated with the presence of AFM1 in bulk tank milk in Argentina.

2. Materials and methods

2.1. Farm selection and sampling

The study was carried out from September 2012 to August 2013. Unit for the statistical analysis was the raw bovine milk samples collected from cooling tanks from different dairy farms (n = 40) located in the Argentina's central dairy region (Santa Fe Province). This area has heterogeneous soils generally suitable for agriculture with medium and high productive capacity. The principal activity is the livestock, mainly dairy based on alfalfa pastures. This area produces 37% of the country's total milk. In this region there are 2894 dairy farms (Ministerio de la Producción, 2010). Sampling took place during four climatic seasons: spring-summer (2012) and autumn-winter (2013); and milk samples were immediately frozen (−18 °C) for further analysis. Milk from one specific farm was employed as blank sample for method development experiments, after checking the absence of AFM1 through IAC-UHPLC-MS/MS analysis.

2.2. Analytical methodology

2.2.1. Reagents

An AFM1 stock standard solution of 10 mg L^{-1} in acetonitrile with a purity of 98.5% was supplied by Supelco (Sigma–Aldrich, Bellefonte, PA, USA). Optima®-grade water, methanol (MeOH) and acetonitrile (MeCN) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium acetate (NH4Ac) (>98%) was obtained from Anedra (Argentina), and formic acid (96%) was obtained from Tedia (Fairfield, OH, USA). Immunoaffinity columns (AFLAPREP® M) were supplied by R-Biopharm Rhône (Glasgow, Scotland).

A 1 mg L^{-1} intermediate standard solution was prepared by dilution of the stock standard solution with MeCN and stored at −18 °C. In the same way, working standard solutions of 100 and 50 μg L^{-1} were prepared from intermediate solution to be used for spiking samples and in the calibration assays.

2.2.2. Equipment and analytical conditions

Ultra Performance Liquid Chromatography was employed using an ACQUITY UPLC™ System (Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer (Micromass TQ Detector from Waters, Manchester, UK) through an orthogonal-Z-spray ionization source. The separations were performed using an ACQUITY UPLC® BEH C18 RP Shield (1.7 μm 2.1 × 100 mm) column from Waters at a flow rate of 0.3 mL min^{-1} and 40 °C temperature. Mobile phase consisted of A (0.5 mM NH4Ac + 0.1% formic acid in water) and B (MeOH 100%) programmed with a time gradient that started at 20% B during 1 min, then linearly increased to 100% B in 1.6 min, remained in pure MeOH for 2.4 min, and finally returned to initial conditions in 1 min. Column was allowed to re-equilibrate during one additional minute before next injection, giving a total run time of 7 min. Injection volume was of 10 μL.

For MS/MS analysis nitrogen was employed as desolvation and cone gas at a flow rate of 900 and 48 L h^{-1}, respectively. The ESI source was operated in positive ion mode with a capillary voltage of 3.2 kV, and 120 °C temperature for the source and 390 °C for desolvation (Chen, Hsu, & Chen, 2011). The cone voltage was 40 V and gas argon (Ar) was used in the collision cell at a flow rate of 0.14 mL min^{-1} (1.3 e−5 mbar) for ion fragmentation. MassLynx v4.1 software (Waters, Manchester, UK) was employed for instrumental operation, data acquisition and analysis.

2.2.3. Preparation of samples

The frozen samples were placed in a fridge overnight to thaw and prior to centrifugation they were warmed for a few minutes in a water bath at 37 °C and homogenized by occasionally gently inverting the containers by hand. Homogenized milk was transferred to suitable tubes and centrifuged for 15 min at 3500 rpm. Skimmed milk was carefully taken from the middle part of the tubes with a syringe and needle, and then filtered through filter paper under vacuum.

For recovery studies defatted milk was spiked with AFM1 working standard solution to yield analyte concentrations 0.05 and 0.1 μg L^{-1}. The spiked milk was gently stirred for 2 h to allow appropriate contact between the added aflatoxin and the matrix.

Immunoaffinity Cleanup (IAC) procedure: 50 mL of defatted milk were loaded into 60-mL syringe barrels placed on top of the IAC columns. Milk was passed through the columns under vacuum at a flow rate of 1–2 drops per second. Then, the columns were washed with two portions of 10 mL of water in order to flush matrix components, and then dried under air for 30 s to avoid dilution of bound analyte. AFM1 was then eluted into amber glass vials with 2 × 1.25 mL aliquots of MeCN:MeOH (60:40). The eluate was filtered through 0.2 μm nylon filters and injected into the UHPLC system with no need of a concentration step.

2.3. Statistic analysis

Each milk farmer surveyed answered an 18-questions structured questionnaire (available upon request) divided into two sections: a) general characteristic of the milk farm (e.g. farm milk

production, number of cows, production per cow, etc.), and b) potential factors associated with the presence of AFM1 in milk (i.e. consumption of different feedstuff, the knowledge that dairy farmers had about mycotoxins, the use of sequestering agents). The purpose and importance of the survey was previously explained to the farmers, emphasizing that responses should be anonymous, since the interest was not the experience of any particular owner, but the frequency of events at the population level.

To quantify the risk associated with the presence of AFM1 in milk samples (detected/non-detected), the analysis was performed in two stages. First, all variables (general and specific questions) were compared with the dependent variable (bivariate analysis) using T-student test, Mann-Whitney test, $\chi 2$ or Fisher exact test, considering the characteristic of the independent variable. In second place, logistic regression (multivariable analysis) was performed. The estimation method was maximum likelihood with a convergence criterion of 0.01 to a maximum of 10 iterations. Only variables associated with the dependent variable in the bivariate analysis ($P < 0.15$) were offered to the model (Hosmer & Lemeshow, 1989). Colinearity between the selected variables was assessed by calculation of Spearman rank correlations (r). When two potential risk factors were associated ($r > 0.6$), only one was used in the multivariable analysis (i.e. the one with the smallest *P*-value in the univariate analysis). All statistical analyses were carried out using InfoStat software (Universidad Nacional de Córdoba) (Di Rienzo et al., 2012).

3. Results and discussion

3.1. Validation of analytical procedure

The IAC procedure described before was basically the same provided by the column's manufacturer. The only modification introduced was the use of water instead of a saline buffer (PBS) in the washing step. This was due to some precipitation observed in the vials when AFM1 was eluted off the column with the methanol-acetonitrile mixture after washing with PBS. When washing the columns with water such precipitation was not observed. Also, the amount of solvent was tested to be sufficient to fully elute the mycotoxin. An extra 1 mL-aliquot of elution solvent was passed through the column, collected in a separate vial and injected to the chromatographic system. No peaks were obtained, indicating that all AFM1 is eluted with two aliquots of 1.25 mL. During the passage of solvent through the column backflush was carried out to ensure a complete breakage of the aflatoxin-antibody bond and to improve recovery rates.

The performance of the IAC extraction was evaluated through recovery studies by spiking defatted blank raw milk with AFM1 standards at 0.10 and 0.05 μg L^{-1}. Focus was put on the lower level since it is the MRL set by European Union regulations. The precision of the method was evaluated by the relative standard deviations (RSD) from intra-day (repeatability) and inter-day (reproducibility) replicates. All recovery and RSD values were in accordance with regulations for mycotoxin analysis (2002/657/EC guidelines). The recovery results at 0.05 μg L^{-1} were %REC = 98% (n = 10) with intra-day precision RSD = 7.5% (n = 4) and inter-day variability RSD = 18% (n = 10) respectively. The second spiking level (0.10 μg L^{-1}) showed similar adequate results (%REC = 99%, intra-day RSD = 1.4%, and inter-day RSD = 15%).

The calibration was evaluated with AFM1 standards in solvent and in the presence of matrix (IAC extracts from blank samples). The linearity was assessed in the range 0.1–100 μg L^{-1} in solvent and 0.5–10 μg L^{-1} in matrix obtaining regression coefficients (r^2) always greater than 0.990 in both cases. The matrix effect (ME) was evaluated by comparing calibration slopes (ME = matrix-matched slope/solvent slope × 100) resulting in an average ME value of 85% (n = 10). This means a 15% of signal decrease comparing with calibration in solvent and shows the AFM1-specificity of IAC columns to the detriment of other matrix components that are washed out.

The limits of detection (LOD) and quantification (LOQ) were estimated as the lowest analyte concentration that produced S/N = 3 and S/N = 10 for the quantification transition, respectively. In addition, S/N = 3 must be verified for the confirmation transition in both cases. Under these criteria LOD was 0.001 μg L^{-1} and LOQ 0.003 μg L^{-1}. These low values clearly indicate the good capability of the method to comply with MRL specifications worldwide for AFM1 in milk (0.5 μg L^{-1} for MERCOSUR, United States and China, and 0.05 μg kg^{-1} for European Union).

An identification and quantification criteria was established for UHPLC-MS/MS determination. Positive samples were identified and quantified when the following criteria was verified: a) the presence of both quantification (329 > 273) and confirmation (329 > 259) transitions; b) retention time tolerance of ±0.2 min for AFM1 in sample extract compared to the calibration standard and between both transitions; c) S/N ≥ 10 for quantification trace (Q) and S/N ≥ 3 for confirmation trace (q) at the same time; d) q/Q ratio tolerance of ≤30% for the sample extract compared to a standard in matrix (SANCO/12571/2013) (Fig. 1).

3.2. Descriptive analysis of experimental assays

The milk samples (n = 160) were taken from 40 dairy farms in each season. The average herd size of these dairy farms was 161 dairy cows (range = 70–407 cows) and a daily milk production of 3623 L (range = 1400–11800). The daily milk production per cow was, on average, 21.80 L (range = 15–32 L).

The herds received a diet based on pastures (essentially alfalfa), corn silage and grains (e.g. corn, sorghum, cotton seed) as source of protein or carbohydrates. However, this diet was variable according to the season. Alfalfa was the main feedstuff used in the diet during spring and summer, while during the rest of the year, oatmeal pasture was also included. Alfalfa hay and sorghum were other sources of fiber used in the milk farms. As sources of protein and energy, the most commonly used ingredients were: soybean expeller, cotton seed, corn, and corn silage.

Sixty two from 160 samples (38.8%) were contaminated with AFM1. The detected AFM1 levels ranged from 0.003 μg L^{-1} (LOQ) to 0.293 μg L^{-1}, with a mean of 0.037 μg L^{-1}. Fifty out of 62 (80.6%) samples which showed detectable levels of AFM1, had concentrations below the maximum level established by the European regulations (0.05 μg L^{-1}), (EC N° 1881/2006) but all the milk samples were within the acceptable limit of AFM1 defined for fluid milk by Southern Common Market (MERCOSUR) Technical Regulations (MERCOSUR GMC/RES. N° 25/02).

3.3. Bivariate analysis

The prevalence of AFM1 in bulk tank milk was not modified by season ($P = 0.617$). The characteristics of the milk farm were not related with the likelihood of AFM1 presence in milk. Neither the number of cow per farm ($P = 0.183$), the total amount of milk produced per farm ($P = 0.213$) nor the milk production per cow ($P = 0.498$) were associated with the presence of AFM1 in milk. However, the milk farms whose cows produced on average more than 21.5 L of milk a day had grater probability to show AFM1 in the milk ($P = 0.084$) (Tables 1 and 2). The number of cows per milk farm and the total amount of milk produced were highly correlated ($r = 0.950$).

Regarding the feed used in each milk farm, pasture silage

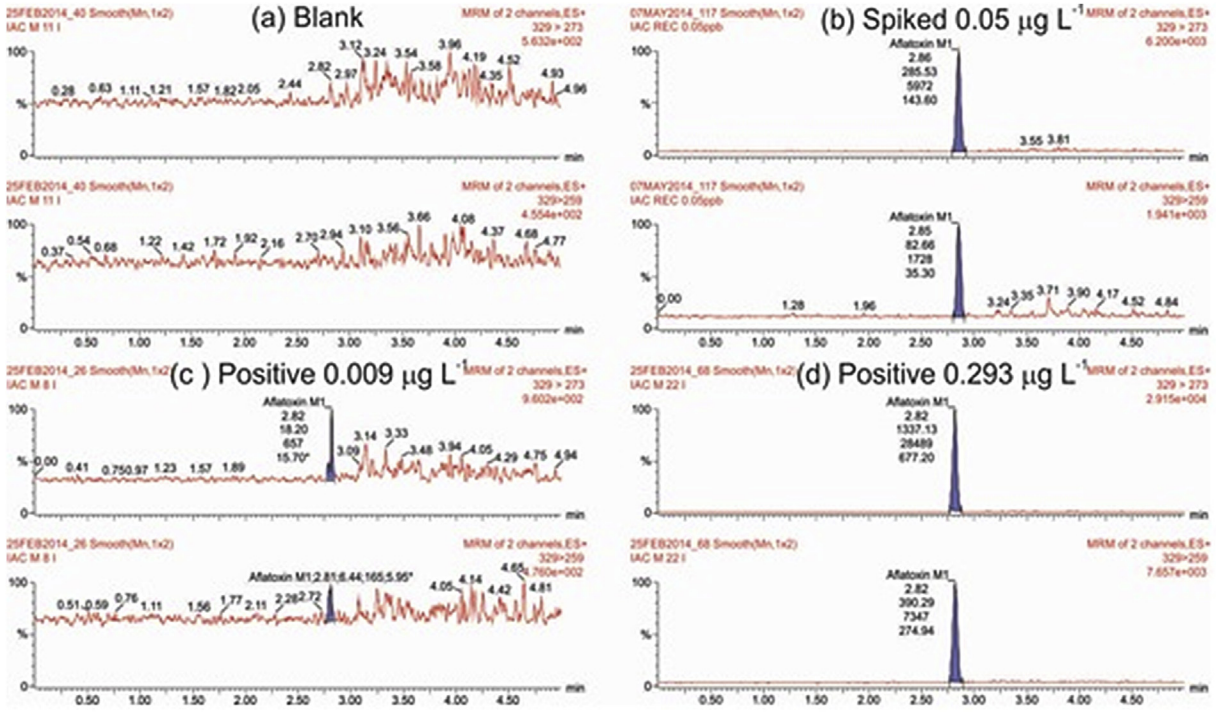

Fig. 1. Corresponding chromatograms of (a) Blank sample, (b) Spiked sample at 0.05 μg L^{-1}, (c) Positive sample with AFM1 concentration near the LOQ (0.009 μg L^{-1}), (d) Positive sample with high AFM1 concentration (0.293 μg L^{-1}).

($P = 0.648$), oatmeal ($P = 0.897$), milk permeate ($P = 0.317$), hay ($P = 0.771$), corn or sorghum grains ($P = 0.338$), corn silage ($P = 0.915$) and soybean expeller consumption ($P = 0.198$), were not associated with the presence of AFM1 in bulk tank milk.

However, alfalfa pasture ($P = 0.121$), commercial feed ($P = 0.017$), and cotton seed consumption ($P = 0.030$) were associated with the prevalence of this mycotoxin in milk (Table 1). In these cases, the presence of AFM1 in milk was not only associated with the consumption of these feedstuffs but also with the amount of each feed in the diet. The amount of soybean expeller ($P = 0.082$) and cotton seed included in the diet ($P = 0.023$) were associated with the presence of AFM1 in the bulk tank milk (Table 2). Thus, the amounts of these ingredients were categorized considering the distribution frequency of the consumption (Table 1).

Finally, the variables offered to the regression logistic model were: milk production per cow, alfalfa consumption, soybean expeller consumption per cow, commercial feed consumption, and cotton seed consumption per cow.

3.4. Multivariable analysis

Some ingredients used in the diet had significant effect on the presence of AFM1 in raw milk. When farmers added more than 0.95 kgDM/cow of soybean expeller in the diets, the presence of AFM1 in raw milk increased (OR = 3.542, $P = 0.019$). When the diets included commercial feed the risk of AFM1 presence in milk was 4.630 times higher than in milk farms that did not add commercial feed in the diet ($P = 0.001$). Moreover, the probability of AFM1 contamination was significantly increased if cows were fed with more than 1.5 kg of cotton seed (OR = 2.944, $P = 0.089$) (Table 3).

On the other hand, alfalfa pasture consumption and milk production per cow were not associated with the presence of AFM1 in raw milk. The dairy farms that used alfalfa pastures as the basis of the diet were also those which tended not to feed their cows with cottonseed ($P < 0.001$) and also used soybean expellers in the diets ($P < 0.001$). Moreover, milk farms whose cows produced more than 21.5 L of milk were those that used soybean expellers in the cows' diet ($P < 0.001$). Therefore, the greater the consumption of cotton seed, the lower the consumption of soybean expellers, and thus the higher the probability of AFM1 presence in raw milk. Noteworthy, the milk farms with higher milk production were those that added soybean expellers in the cows' diet.

3.5. Prevalence and associate risk factors

The total prevalence of AFM1 in milk from bulk tanks in farms located in the most important milk-production zone in Argentina was important (38.8%). The average level of AFM1 in bovine milk was within the maximum level accepted by the MERCOSUR regulation (0.5 μg L^{-1}) (MERCOSUR GMC/RES. N° 25/02) and 80.6% of the samples showed levels lower than the limit established by the European regulations (0.05 μg kg^{-1}) (EC N° 1881/2006, EC N° 165/2010).

The studies in Argentina are controversial. Some authors reported that 64% of the bulk tank milk samples were contaminated with AFM1 (Alonso et al., 2010), while other authors reported incidences of 10.8% in farm milk (López et al., 2003). Another study conducted in the Argentina's central dairy region (Basílico & Zapata de Basílico, 2005), identified that out of 33 samples of raw milk, only two of them had detectable levels of AFM1. The three studies were conducted using comparable methodologies with the same LOD (0.01 μg L^{-1}). However, in these studies all AFM1 concentrations were below the maximum tolerated levels established by MERCOSUR legislation. These studies were conducted in different dairy regions in Argentina, so those differences could be considered a consequence of different ingredients (especially concentrated feeds and pasture), and/or quantities in the diets. The proportion in which these different feed sources are used in the diet of dairy

Table 1
Definitions and distributions of explanatory variables (categorical) included in the analysis for potential association with the presence of AFM1 in raw milk, Santa Fe province (Argentina) (n = 160 milk farms).

Variable	Level	Size	% Positive	*P*-value
Season	Spring	40	35.0	0.617
	Summer	40	47.5	
	Autumn	40	37.5	
	Winter	40	35.0	
Milk production per cow	<21.5 lts/cow	75	32.0	0.084
	>21.5 lts/cow	79	45.6	
Total milk production	<3000 L	77	35.1	0.426
	>3000 L	75	41.3	
Alfalfa consumption	No	16	56.3	0.121
	Yes	127	36.2	
Pasture silage	No	107	37.4	0.648
	Yes	36	41.7	
Oatmeal consumption	No	128	38.3	0.897
	Yes	15	40.0	
Permeate	No	133	39.8	0.213
	Yes	10	20.0	
Hay	No	68	39.7	0.771
	Yes	75	37.3	
Grain	No	63	42.9	0.338
	Yes	80	35.0	
Silage	No	24	37.5	0.915
	Yes	119	38.7	
Soybean Expeller	No	95	34.7	0.198
	Yes	48	45.8	
Soybean expeller consumption per cow	<0.95 kgDM/cow	108	35.2	0.082
	>0.95 kgDM/cow	37	51.4	
Commercial feed	No	70	28.6	0.017
	Yes	73	47.9	
Cotton seed	No	110	33.6	0.030
	Yes	33	54.5	
Cotton seed consumption per cow	<1.5 kgDM/cow	128	35.9	0.023
	>1.5 kgDM/cow	17	64.7	

Table 2
Definitions and distributions of explanatory variables (continuous) included in the analysis for potential association with the presence of AFM1 in raw milk, Santa Fe province (Argentina) (n = 160 milk farms).

Independent variable	Mean on negative dairy farms	Mean on positive dairy farm	*P*-value
Number of cows	155.03	171.76	0.183
Milk production per dairy farm	3460.81	3886.83	0.213
Milk production per cow	21.66	22.04	0.498
Alfalfa consumption (kg DM/cow)	3.34	3.10	0.656
Oatmeal consumption (kg DM/cow)	0.19	0.28	0.557
Hay consumption (kg DM/cow)	0.82	0.65	0.280
Corn grain consumption (kg DM/cow)	2.57	2.09	0.268
Silage consumption (kg DM/cow)	2.69	2.98	0.572
Pasture silage (kg DM/cow)	1.19	1.71	0.311
Soybean expeller consumption (kg DM/cow)	0.58	0.92	0.123
Commercial feed consumption (kg DM/cow)	2.05	2.96	0.049
Cotton seed consumption (kg DM/cow)	0.24	0.49	0.036

Table 3
Logistic regression of risk factors associated with AFM1 contamination in bulk tank milk (n = 160, Santa Fe province, 2013).

Predictive variables	B	SE	P	OR	OR CI 95%
Constant	−1.778	0.430	<0.001	–	–
Cotton seed cons./cow	1.080	0.634	0.089	2.949	0.850–10.203
Soybean expeller cons./cow	1.265	0.533	0.019	3.542	1.234–10.167
Commercial feed cons.	1.533	0.473	0.001	4.603	1.831–11.708

Hosmer-Lemenshow P = 0.895.
Reference populations: Soybean expeller consumption per cow <0.95 kgDM/cow, Commercial feed consumption No, Cotton seed consumption <1.5 kgDM/cow.
SE:Standard Error. OR: Odds Ratio. CI Confidence Interval.

cattle varies considerably and is determined by regional differences, the production stage of the animal, and farm management (Fink-Gremmels, 2008).

However, the studies conducted in different areas in Argentina agree that the positive samples had very low levels of AFM1. The study conducted by Basílico and Zapata de Basílico (2005), identified that AFM1-contaminated milk samples had concentration <1 μg L^{-1} Alonso et al. (2010) reported that levels of detected AFM1 ranged from not detectable to 0.07 μg L^{-1}. Moreover, López et al. (2003) showed that the average level of AFM1 in farm milk was 0.016 μg L^{-1}. In Argentina, the National Plan for Residue Management and Food Safety (CREHA) reported that, from 2003 through 2014, 1159 out of 2862 samples (40.49%) of raw milk in dairy industry were AFM1-contaminated, with values between 0.025 μg L^{-1} and 0.5 μg L^{-1}; and three samples (0.10%) showed values higher than 0.5 μg L^{-1} (CREHA, 2012). Even considering the variable incidence of AFM1-contaminated milk in those studies, the levels of AFM1 were similar to the concentrations observed in this

study.

The milk farms located in Argentina's central area had a semi-intensive system of breeding and an important proportion of the diet is constituted by concentrated feeds (eg. soybean expellers, cotton seed) and corn silage. These types of ingredients have been highly correlated with the level of mycotoxins in dairy milk in a previous quantitative risk assessment (Signorini et al., 2012). The use of these ingredients is most used in high-intensive farms. Genetic selection for high milk yield needs an increase in the quantities of digestible energy-rich feed ingredients to the diet (Fink-Gremmels, 2008). These types of feedstuff, especially cotton seed (Alonso et al., 2010), have a higher risk of being contaminated with AFB1. In this study, commercial feed, cotton seed, and soybean expeller were identified as risk factors for the presence of AFM1 in raw milk. Additionally, milk farms that used these types of ingredients in the diet of cows were characterized by a more intensive production, and therefore showed a higher risk of presence of AFM1 in milk. The relationship between the prevalence of AFM1 in milk and the milk yield could also be explained by the direct effect of milk yield on the total AFM1 excretion as was suggested by Masoero, Gallo, Moschini, Piva, and Díaz (2007) and Veldman, Meijs, Borggreve, and Heeres van der Tol (1992).

A previous quantitative risk assessment (Signorini et al., 2012) performed in the same region from Argentina predicted that approximately 0.66% of the milk produced in Argentina's central dairy region exceeds the maximum level accepted by MERCOSUR. However, considering the European regulations, the estimated percentage of samples that exceed the maximum were 32.65%. In this study none of the samples showed levels of AFM1 higher than the maximum level accepted by MERCOSUR and only 7.5% of the milk samples presented levels of AFM1 higher than the limit established by the European regulation. Therefore, we may conclude that the estimations provided by quantitative risk assessments are reliable when compared with the data emerging from field studies.

4. Conclusions

The prevalence and the levels of AFM1 in milk produced and commercialized in Argentina is not serious. Cotton seed, soybean expeller, and commercial feed seem to be the highest-risk ingredients when applied in dairy cattle feed. Since the quality requirements are increasingly stringent, any reduction in established international regulatory limits would be a serious impact on domestic production.

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References

Alonso, V. A., Monge, M. P., Larriestra, A., Dalcero, A. M., Cavaglieri, L. R., & Chiacchiera, S. M. (2010). Naturally occurring aflatoxin M_1 in raw bulk milk from farm cooling tanks in Argentina. *Food Additives & Contaminants: Part A, 27*, 373–379.

Basílico, J. C., & Zapata de Basílico, M. L. (2005). Aflatoxina M_1 [Aflatoxin M_1]. In INTA (Ed.), *Manual de referencias técnicas para el logro de leche de calidad [Handbook of technical reference for achieving quality milk]* (pp. 63–68). Buenos Aires (Argentina): Instituto Nacional de Tecnología Agropecuaria.

Binder, E. M. (2007). Managing the risk of mycotoxins in modern feed production. *Animal Feed Science and Technology, 133*, 149–166.

Black, R. D., McVey, D. S., & Oehme, F. W. (1992). Immunotoxicity in the bovine animal. *Veterinary and Human Toxicology, 34*, 438–442.

Chen, W. L., Hsu, T. F., & Chen, C. Y. (2011). Measurement of aflatoxin M1 in milk by ultra-high-performance liquid chromatography/tandem mass spectrometry. *Journal of AOAC International, 94*(3), 872–877.

CREHA. (2012). *Plan Nacional de Control de Residuos e Higiene en Alimentos. Resumen de resultados 2012. [National Plan for Residue Management and Food Safety. Abstract 2012].* http://www.senasa.gov.ar/Archivos/File/File6900Resumen_resultados_Plan_CREHA_2012.pdf (last accessed on March 2014).

Creppy, E. E. (2002). Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicology Letters, 127*, 19–28.

Di Rienzo, J. A., Casanoves, F., Balzarini, M. G., Gonzalez, L., Tablada, M., & Robledo, C. W., InfoStat version 2012. InfoStat group, FCA, Cordoba National University, Argentina. URL http://www.infostat.com.ar.

D. SANCO/12571/2013. Guidance document on analytical quality control and validation procedures for pesticide residues analysis in food and feed. http://www.eurlpesticides.eu/library/docs/allcrl/AqcGuidance_Sanco_2013_12571.pdf (last accessed in March, 2014).

EU. (2002). Commission decision 2002/657/EC of 12 of August 2002, concerning the performance of analytical methods and the interpretation of the results. *Official Journal of the European Communities, 2002*, L221–L232.

European Community. (2006). *Commission regulation No 1881/2006 of 19 December 2006, setting maximum levels for certain contaminants in foodstuffs*.

European Community. (2010). Commission Regulation (EC) No.165/2010 of 26 February 2010 Amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. *Official Journal of European Communities, L50*, 8–12.

Fink-Gremmels, J. (2008). Mycotoxins in cattle feeds and carry-over to dairy milk: a review. *Food Additives and Contaminants, 25*, 172–180.

Hosmer, D. W., & Lemeshow, S. (1989). *Applied logistic regression*. New York: John Wiley & Sons. Inc, 307pp.

López, C. E., Ramos, L. L., Ramadan, S. S., & Bulacio, L. C. (2003). Presence of aflatoxin M1 in milk for human consumption in Argentina. *Food Control, 14*, 31–34.

Masoero, F., Gallo, A., Moschini, M., Piva, G., & Díaz, D. (2007). Carryover of aflatoxin from feed to milk in dairy cows with low or high somatic cell counts. *Animal, 1*, 1344–1350.

ERCOSUR/GMC Resolution 25/02. (2002). *Reglamento técnico MERCOSUR sobre límites máximos de aflatoxinas admisibles en leche, maní y maíz [MERCOSUR technical regulation about maximum tolerable limits for aflatoxin in milk, peanuts, and corn].* www.mercosur.int (last accessed in March 2014).

Ministerio de la Producción [Ministry of Production]. (2010). Sistema Sanitario Productivo y Participativo [Productive and participatory health system]. Santa Fe (Argentina). In *Gobierno de la Provincia de Santa Fe*.

Picinin, L. C. A., Oliveira, M. M., Azevedo, V. E., Quintao, L. A. M., Toaldo, I. M., & Bordignon-Luiz, M. T. (2013). Influence of climate conditions on aflatoxin M1 contamination in raw milk from Mina Gerais State, Brazil. *Food Control, 31*, 419–424.

Prandini, A., Tansini, G., Sigolo, S., Filippi, l., Laporta, M., & Piva, G. (2009). On the occurrence of aflatoxin M_1 in milk and dairy products. *Food Chemical Toxicology, 47*, 984–991.

Scaglioni, P. T., Becker-Algeri, T., Drunkler, D., & Badiale-Furlong, E. (2014). Aflatoxin B1 and M1 in milk. *Analytica Chimica Acta, 829*, 68–74.

Signorini, M. L., Gaggiotti, M., Molineri, A., Chiericatti, C. A., Zapata de Basílico, M. L., Basílico, J. C., et al. (2012). Exposure assessment of mycotoxins in cow's milk in Argentina. *Food and Chemical Toxicology, 50*, 250–257.

Van Egmond, H. P. (1989). In *Mycotoxins in dairy products*. London (UK): Elsevier Applied Science.

Veldman, A., Meijs, J. A. C., Borggreve, G. J., & Heeres van der Tol, J. J. (1992). Carry-over of aflatoxin from cows food to milk. *Animal Production, 55*, 163–168.