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Incidence of Mycotoxins (AFB_1 and AFM_1) in Feeds and Dairy Farms from Rio de Janeiro State, Brazil

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ABSTRACT

Brazil has regions located at the largest dairy production and milk derivate industry concentration, supplying the major consumer markets, represented by São Paulo, Rio de Janeiro and Belo Horizonte Cities. The milk is the most important product of Brazilian agriculture, because it is always presents in daily diet. The aim of this research was evaluating the occurrence of mycotoxins in the feed of dairy cattle and the occurrence of AFM_1 in milk under field conditions in farms from Rio de Janeiro State, Brazil. The results revealed that, total fungal counts was found in 67% of feed samples which exceeded the recommended limit recommended (1×10^4 UFC.g^{-1}) and the incidence of AFM_1 in all milk samples was 26.7% at concentrations ranging from 0.010 to 1.500 µg.L^{-1}. The study revealed toxigenic fungi and their mycotoxins were present in feed intended for bovine feeding in Rio de Janeiro farms. Evaluations of mycotoxin levels are important to provide information so that the assessments of risk for animal feed and livestock environment can be done.

KEYWORDS: Mycotoxins; Aflatoxin M_1; Brazil feeds; Dairy farms; Toxigenic fungi.

ABBREVIATIONS: Afs: Aflatoxins; IARC: International Agency for Research on Cancer; DRBC: Dichloran Rose Bengal Chloranphenicol; CFU: Colony-forming units; MEA: Malt Extract Agar; CLA: Carnation Leaf Agar; OTA: Ochratoxin A; HPLC: High Performance Liquid Chromatography.

INTRODUCTION

Brazil is a largest milk producer, growing at annual rate of 4% and higher than in countries occupying the first positions: It generates 66% of the total volume of milk produced in the countries comprising the MERCOSUR.[1] The country has regions that are located at the largest dairy production and milk derivate industry concentration, supplying the major consumer markets, represented by São Paulo, Rio de Janeiro and Belo Horizonte Cities. The milk is the most important product of Brazilian agriculture, because it is always presents in daily diet, enhancing

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the quest for product quality shall be continuous.[2]

Mycotoxins are metabolites produced by certain species of filamentous fungus and can cause various toxic effects in animals.[3] Human and animal exposure to aflatoxins (AFs) can occur primarily by ingesting contaminated food and feed, mainly cereals and grains, such as corn, wheat, and peanuts, among others. Eighteen (18) different types of AFs were identified, but only aflatoxin B_1(AFB_1), B_2(AFB_2), G_1(AFG_1) and G_2(AFG_2) were detected as natural contaminants and feed ingredients and AFB_1 toxin has a great toxicity.[4-6] A continuous intake of AFB_1 for lactating animals leads to excretion of aflatoxin M_1(AFM_1).[7] Toxic effects related to this carcinogen have been extensively demonstrated,[8] therefore, the International Agency for Research on Cancer (IARC)[9] classified it as a probable human carcinogen factor.

The occurrence of AFM_1 in pasteurized milk in Brazil and MERCOSUR is variable,[10-15] but the observed levels are usually below the tolerance limit of 0.5 $\mu g.L^{-1}$ determined by current standards.[16] The occurrence of AFB_1 and other mycotoxins in feed ingredients for dairy cattle is relatively common in Brazil.[17-20] However, there are few studies on the incidence of AFM_1 in milk in South America, mainly in milk Brazilian production regions.

The aim of this study was to evaluate the occurrence of mycotoxins in the feed of dairy cattle and the occurrence of AFM_1 in milk under field conditions in farms from Rio de Janeiro State, Brazil.

MATERIALS AND METHODS

Sampling

The samples were collected in Rio de Janeiro State, from January 2011 to 2014 in basins that produce milk at different scales (≤150 L/day, from 150-300 L/day and ≥300 L/day), from the prevalent areas of milk production. The periods established for sampling were two semi-annual collections in order to evaluate the seasonal influences in samples. A total of 240 raw milk samples (80 samples of each production level), 120 pasteurized milk samples and 160 samples of feed provided to the animal, were collected.

All sampled dairy farms used diets with soybean and corn, purchased and stored locally. Other ingredients included cottonseed, sorghum silage, forages, corn silage, sugar cane, citrus pulp, barley and wheat. Five aliquots of 500 g of feed were collected from different points of the trough feeding, homogenized and used sterile polypropylenes packaging for a final sample of each basin. Samples for AFM_1 determination were collected during milking of the animals, from the milk cooling tank and after process of pasteurization on the industrial process. Two sterile vials with 500 mL of milk were collected to compose the final sample for each basin from selected region. These samples were transported in an isothermal box to laboratories of PESAGRO-RJ and Núcelo de Pesquisa Micológica e Micotoxicológica (NPMM) da UFRRJ for final (24 h).

Physical Evaluation of the Samples

Dry matter percentage (DM%) and pH of the samples were evaluated[21] and Water activity (a_w) was determined using an AQUALAB CX2 (Decagon Devices, Inc., USA) appliance.

Mycological Survey

Total fungal counts from each sample were performed onto three different culture media: Dichloran Rose Bengal Chloranphenicol (DRBC) agar, a general medium used for estimating total culturable mycobiota[22]; Dichloran 18% Glycerol agar (DG18), a low a_w medium that favours xerophilic fungi development[23]; and Nash and Snyder agar (NSA), a selective medium for *Fusarium spp*. counts.[24] Quantitative enumeration was done using the surface-spread method. Ten grams of each sample were homogenized in 90 mL 0.1% peptone water solution for 30 min in an orbital shaker. Serial dilutions (10^{-2} to 10^{-3}) were made and 0.1 mL aliquots were inoculated in duplicates onto the culture media. Plates were incubated at 25 °C for 7-10 days in darkness. Nash-Snyder plates were incubated at 24 °C for 7 days under a 12 h cold white/12 h black fluorescent light photoperiod. Only plates containing 10-100 colony-forming units (CFU) were used for counting. The results were expressed as CFU per gram of sample ($CFU.g^{-1}$). Representative colonies of *Aspergillus* and *Penicillium spp*. were transferred for sub-culturing to tubes containing malt extract agar (MEA) and *Fusarium spp.* were transferred to carnation leaf agar (CLA). Fungal species were identified according to taxonomic specific protocols.[25-27] The results were expressed as isolation frequency of the fungal genera (% of samples in which each genera was present) and relative density of each fungal species (% of isolation of each species among strains of the same genera).[23]

Toxigenic Profile of Fungal Isolates

The ability to produce ochratoxin A (OTA) by potentially producer strains isolated from samples (*A. carbonarius*, *A. niger* aggregate and *A. ochraceus*) was tested.[28] Aflatoxins production was evaluated in all *Aspergillus* section *Flavi* isolates.[29] *Fusarium*-toxins: primarily fumonisin B_1(FB_1) and zearalenone (ZEA), produced by the isolates.[30]

Mycotoxins Analysis in Feed

For mycotoxins determination in the feed, the samples were evaluated for screening method to qualify toxigenic conditions and make a quantitative previous evaluation. The Vicam® fluorometer (4ex series) engaged with specific immunoaffinity columns (Vicam®, Watertown, MA, USA). All positive samples were confirmed by a High Performance Liquid Chromatography (HPLC) evaluation.

The AFB_1 determination in the feed samples was done

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in parallel of screening method, following the procedures recommended by the manufacturer of solid phase and cleanup phase columns Mycosep (Romer® Labs, Inc., Union, MO, USA) and evaluated by HPLC.

All feed samples were previously crushed and homogenized. Then, the analytical sample was placed in a blender along with specific solvent. The extract was filtered and collected for passage through the columns. The separation and quantification of mycotoxins was conducted on a HPLC system (JASCO LC 2000, Tokyo, Japan) equipped with a fluorescence detector (excitation: 360 nm and emission: 440 nm). The quantification of mycotoxins in the samples was performed by interpolation of the areas of the chromatographic peaks obtained in the samples in the calibration curve regression equation with specific external standards (Sigma-Aldrich, St. Louis, MO, USA).

Analysis of aflatoxin M_1: The extraction and purification of samples for the determination of AFM_1 were performed in duplicate according to the recommendations,[31] with adjustments proposed by the manufacturer of immunoaffinity columns (Aflatest®, Vicam, Watertown, MA, USA) as described by Oliveira et al.[32] In summary, the analytical sample (25 mL) was preheated to 37 °C, added with 1 g of NaCl, and subject to centrifugation (2.500 g, 15 min), after which it was directly passed through immunoaffinity column connected to a vacuum system (flow 2-3 $mL.min^{-1}$). After the sample elution, the column was washed by passing 20 mL of ultrapure water (Milli Q, Millipore) and methanol (9:1, $v.v^{-1}$). The final purified elute was diluted with ultrapure water to form a solution of methanol-water (7:3, $v.v^{-1}$), similar to the HPLC mobile phase. The identification and quantification of AFM_1 residues were conducted with the injection of 20 µL of the extracts of the samples in the HPLC system (JASCO LC 2000) of methanol-water (7:3, $v.v^{-1}$) at a flow rate of 0.8 $mL.min^{-1}$. Under these conditions, the retention time for approximately 3.7 min. The calibration curve was prepared using AFM_1 standard (Sigma, St. Louis, MO, USA) previously solutions evaluated according to Scott (1990), at doses of 0.5, 1.0, 2.5, 5.0 and 10.0 $ng.mL^{-1}$.

Statistical Analysis

Data analysis were performed by analysis of variance (ANOVA). The test of least-significant differences (LSD) was used to determine the significant differences between means. Analysis was conducted using PROC GLM in SAS (SAS Institute, Cary, NC, USA). Statistical significance was indicated by $p \leq 0.05$.

RESULTS

Physical evaluation of the samples did not show significant differences in DM%, pH and a_W values for seasonal samples. The mean (DM%) of 47.17±5.76%, pH values varied from 3.88 to 4.89 and a_W varying between 0.729 and 0.985.

The total Fungal Counts are specified at Table 1 ($7.3*10^5$-$1.4*10^3$ $CFU.g^{-1}$). The profiles of isolated strains, Table 2, approximately sixty percent of *A. flavus* and *A. parasiticus* (79 out of 136 isolates), were able to produce AFB_1 and AFB_2 at ranges from 0.02 to 25.0 $\mu g.kg^{-1}$. Of twenty five strains of *A. niger* aggregate isolates (23%) showed ability to produce at ranges from 0.05 to 10.0 $\mu g.g^{-1}$ of OTA. All strains of *F. verticillioides* able to produce FB_1 at ranges from 0.2 to 8.0 $\mu g.g^{-1}$.

When the samples of feed contaminated with AFB_1 were compared, the range levels (0.2-50.0 $\mu g.kg^{-1}$) and the frequency of the contaminated milk samples showed in 75% with AFM_1 at range levels (0.05-1.50 $\mu g.L^{-1}$). The quantification limits for AFB_1 and AFM_1 were 0.02 and 0.05 $\mu g.L^{-1}$, respectively, considering the minimum amount of toxin that could produce a chromatographic peak three times the baseline standard deviation. The Table 2 shows data on milk yield level, the number of posi-

Samples	Season	Total fungal counts (CFU g^{-1}) Mean ± SD	
		Culture media	
		DRBC	DG18
Corn and Corn meal	Su	$5.8x10^5 \pm 1.0x10^{4\ ab}$	$5.8x10^4 \pm 1.0x10^{4\ ab}$
	Au	$4.3x10^4 \pm 1.6x10^{4\ a}$	$4.3x10^4 \pm 1.6x10^{4\ a}$
	Wi	$3.4x10^5 \pm 1.5x10^{4\ b}$	$4.4x10^5 \pm 1.5x10^{4\ b}$
	Sp	$5.7x10^5 \pm 1.5x10^{4\ b}$	$5.7x10^5 \pm 1.5x10^{4\ b}$
Corn Silage and Wheat Brew Silage	Su	$7.3x10^5 \pm 2.8x10^{5\ a}$	$3.4x10^4 \pm 1.4x10^{4\ a}$
	Au	$9.2x10^4 \pm 9.1x10^{3\ ab}$	$3.6x10^4 \pm 1.0x10^{4\ a}$
	Wi	$7.3x10^4 \pm 1.0 \times 10^{4\ ab}$	$3.6x10^4 \pm 1.3x10^{4\ a}$
	Sp	$1.3x10^4 \pm 8.5x10^{3a}$	$3.8x10^4 \pm 1.1x10^{4\ a}$

*Referring to DRBC culture medium; SD: Standard deviation; Detection limit: 10^2 $CFU.g^{-1}$. ^{a,b} Values indicated with different letters are significantly different according to LSD test ($p<0.05$ for seasonal period samples). Su: Summer, Au: autumn, Wi: winter, Sp: spring.

Table 1: Total fungal counts (CFU g^{-1}) found in feed samples collected from different farms in four (4) seasonal periods of the year and evaluated on DRBC and DG18 media.

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Milk production scale	Season	Aflatoxin levels range (μgKg^{-1}/μgL^{-1})					
		AFB_1		AFM_1 (no processed milk)		AFM_1 (processed milk)	
		%	Mean±SD	%	Mean±SD	%	Mean±SD
≤150 L/ day	Su	100	1.1±0.2	12	0.039±0.01	10	0.03±0.01
	Au	100	3.4±0.3	13	0.041±0.01	11	0.01±0.01
	Wi	100	4.1±0.2	16	0.042±0.01	10	0.02±0.01
	Sp	100	2.2±0.3	14	0.031±0.01	14	0.01±0.01
150-300 L/day	Su	100	5.5±1.5	24	0.28±0.12	19	0.02±0.01
	Au	100	6.3±1.3	26	0.31±0.13	17	0.04±0.01
	Wi	100	4.2±1.1	30	0.52±0.21	29	0.04±0.02
	Sp	100	5.2±1.2	28	0.41±0.21	27	0.03±0.01
300 L/ day	Su	100	5.9±1.5	44	0.29±0.22	34	0.03±0.01
	Au	100	7.3±1.3	42	0.41±0.10	32	0.04±0.01
	Wi	100	5.2±1.1	49	0.62±0.13	31	0.06±0.01
	Sp	100	6.2±1.2	47	0.41±0.21	32	0.04±0.02

*SD: Standard deviation; Detection limit: AFB_1 and AFM_1 were 0.02 and 0.10 μg.L^{-1}; Su: Summer; Au: autumn; Wi: winter; Sp: spring.

Table 2: Incidence range of aflatoxin B_1 and M_1 in farms of Rio de Janeiro State and categorized on three milk production scales.

tive samples, the range and the average concentrations of AFs in the milk and the animal feed samples.

The incidence of AFM_1 in all milk samples was 25.45% at concentrations ranging from 0.05 to 1.500 μg.L^{-1}. This frequency was consistent with the detection of AFM_1 at all times of milk samples selected. Farm samples with milk production between 150 and 300 L/day showed also positive samples for AFM_1 (38.90%).

DISCUSSION

The results of differences in DM%, pH and a_W values for seasonal samples are comparable to those obtained by Keller et al[33] in Brazil and González Pereyra et al[34] in Argentina.

Total fungal counts present in 67% of feed samples, Table 1, exceeded the limit recommended as quality standard (1×10^4 UFC.g^{-1}) proposed by GMP[16] and Brazilian reglamentation.[35] The total fungi isolated were increased during rainy season and temperature rise similar as those reported for other regions.[36,37] Mycobiota isolated from several feed samples were comparable to species found by other researchers from the same substrate in Brazil, Argentina, France and Egypt being potentially toxigenic species *A. flavus*, *A. parasiticus*, *A. fumigatus*, *A. niger* aggregate, *P. citrinum*, *F. verticillioides* and *F. graminearum* prevailed on this substrate. *Aspergillus flavus* and *A. fumigatus* relative density in post- fermentation silage samples was higher than in pre-fermentation samples.[38-40]

The screening of feed samples allowed evaluating a concurrence of several mycotoxins. The AFB_1 contamination was detected in all samples, areas and seasonal periods, many of them were exceeded the recommended limit for AFB_1 in cattle feed (20 μg.kg^{-1}) proposed by GMP. Also, OTA, FB_1, and ZEA were detected. However, when the increase of the mycotoxin level on severe seasonal conditions was evaluated, the statistical differences were not found.

This evaluation suggests that mycotoxins contamination was enhanced during storage.[41] The ensiling process supposes control fungal contamination since pH is reduced to an extremely acid condition and oxygen is consumed to anaerobiosis. However, bad storing condition and practices during the ensiling process or even after the silo is opened for feeding-out, can lead to this kind of contamination.[42]

The AFB_1 levels observed in the feed samples remained below the tolerance limit recommended (50.0 μg.kg^{-1}) for feed ingredients in Brazil.[43] The research reveals that toxigenic fungi and their mycotoxins are present in feed intended for bovine fed in Rio de Janeiro farms, as occurs in other Brazilian States. Subsequent evaluations of mycotoxin levels are important to provide information, so that the assessments of risk for animal feed and livestock environment can be made.

Sabino et al[44] found 18% positive samples at levels from 0.10 to 1.68 μg.L^{-1} in the San Pablo State. Recently, Sassahara et al[45] and Oliveira et al[46] found AFM_1 at levels from 0.29

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to 1.97 µg.L^{-1} in 24% of raw milk samples collected from farms in the Paraná and São Paulo States, respectively. The process of modernization of the milk chain production in order to conform to current standards regulations in Brazil, has promoted a significant increase in milk production, enabling the export of dairy products. This increase is due in part to the extensive supply of rations to animals, especially in the off season months (autumn and winter), which may have contributed to obtain detectable levels of AFM_1 in milk of the studied regions.

Sampling performed during the months of spring and summer obtained all samples with AFM_1 above 0.5 µg.L^{-1}. However, 10% of the milk samples collected on other mouths had higher levels of AFM_1 tolerance limit adopted by the European Union (0.050 µg.L^{-1}).

This study shows the need to revise the legislation for AFs in rations and AFM_1 in raw milk in Brazil to prevent the occurrence of AFB_1 and other mycotoxins in feed ingredients for dairy cattle and consequently human toxicity. On addition reinforces the importance of the revision of standard, in order to establish consistent limits for mycotoxins in feed ingredients intended for dairy cattle.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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