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Monitoring of Mycotoxins in Feed for Goats and Their Residues in Milk

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Abstract

Aflatoxins are mainly produced by *Aspergillus flavus* and *A. parasiticus*, whereas *A. flavus*, under different conditions, also can produce cyclopiazonic acid (CPA). Several studies on mycotoxins in feed and cow milk have been reported, but the investigation of the co-occurrence of aflatoxins and CPA in feed and goat milk is an unprecedent study in Brazil. This study aimed to evaluate the presence of aflatoxins and cyclopiazonic acid in diets intended for dairy goats and their residues in milk in 10 familiar properties of Southwestern São Paulo region, totalizing 128 samples of feed and 120 samples of milk. Aflatoxins have been extracted and purified in an immunoaffinity column specific for aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) and aflatoxin M1 (AFM1). The analyses for detection and quantification of toxins were performed by high-performance liquid chromatography. From the analyses of the feeds, 2.34% of these were contaminated with aflatoxin at levels ranging from: 3.65 to 36.93 µg/kg for AFB1; 0.35 to 2.46 µg/kg for AFB2; trace to 46.06 µg/ kg for AFG1 and 0.61 to 1.65 µg/ kg for AFG2. One sample showed a concentration of 87.1 µg/kg, above that allowed by Brazilian law. Concerning CPA, 3.12% of the feed samples were positive for it at concentrations ranging from trace to 1.90 µg/kg. AFM1 and CPA were not detected in the milk samples. The results demonstrate that low contamination by mycotoxins in feeds and the absence of AFM1 and CPA in milk indicate best practices in the management and storage of these feeds.

Keywords: cyclopiazonic acid, aflatoxins, dairy goats

1. Introduction

Many agricultural products are invaded by fungi before, during and after harvest; at drying, transport and/or storage. The interaction between water activity and temperature is a most critical determinant for fungal growth and mycotoxin production (Gqaleni et al., 1997). In general, peanuts, corn, cotton seed are among the grains with a higher risk of contamination (Ismail, 2000), and are often invaded before harvest by *A. flavus*, producer of aflatoxins and cyclopiazonic acid (CPA) (Pildain et al., 2004). Aflatoxins (B1, B2, G1, G2 and M1) and CPA may occur naturally in other products, such as rice, cheese, eggs, feed, etc. (Steinhart et al., 1996; Hayashi & Yoshizawa, 2005), and often they are co-contaminants (Lisker et al., 1993). Diseases caused by mycotoxins are characterized by diffuse syndromes, however, presenting injuries in certain organs like liver, kidney, epithelial tissue and central nervous system, depending on the kind of toxin. It is also possible the simultaneous occurrence of two or more mycotoxins, which may lead to the potentiation of the toxic effects over the susceptible organism (Oliveira & Correa, 2010).

Animals that consume feed contaminated with mycotoxins may suffer pathological changes in liver, kidneys, lungs, heart and pancreas and implications on their zootechnical performance (low weight gain and feed conversion), causing a great economic impact on livestock production. Another point concern is the

contamination of animal products, once the mycotoxins and their metabolites can be excreted in milk, meat, and eggs (Dorner et al., 1994; Rosmaninho et al., 2006). Aflatoxin B1 (AFB1) ingested by cattle or goats can be biotransformed in the liver in aflatoxin M1 (AFM1), and hence excreted in milk (Rosemaninho et al., 2006). When an animal eats food contaminated with 0.5% of AFB1, 5.0% of this toxin ingested is biotransformed to AFM1 (Hussein & Brasel, 2001); nevertheless, in goats this percentage is from 0.18% to 3.0% (Virdis et al., 2008).

Although most references in the literature report the presence of aflatoxin M1 in cow milk (Dorner et al., 1994; Galvano et al., 1996; Gonçalez et al., 2004; Oliveira et al, 2008), it is known that this toxin can easily occur in milk of sheep, goats, buffaloes and camels (Galvano et al., 1996; Motawee et al., 2009), but there are few reports about the presence of aflatoxin M1 in goat milk (Oliveira et al., 2007). Co-occurrence between AFM1 and CPA in bovine milk has been the subject of studies by other authors (Oliveira et al., 2008; Rosemaninho et al., 2006), however there are no reports about CPA in goat milk.

The ovine and caprine culture has grown in recent years in the State of São Paulo in Brazil, being the southwestem of São Paulo is the largest producer of goat's milk. Therefore, to check the co-occurrence of AFM1 and CPA in goat milk and its derivatives deserves much attention due to their toxic effects on humans and animals. The present study aimed to monitor the levels of aflatoxins B1, B2, G1 and G2, and CPA in feed intended for dairy goats and their residues in bulk milk from familiar properties located in southwestern São Paulo, Brazil.

2. Material and Methods

2.1 Samples

Samples of feed (500 g each) and milk (250 mL each) were collected in 10 producing properties of goat milk located in southwestern São Paulo: 3 in Ibiúna (P1, P2, and P3), 2 in Piedade (P4 and P5), 1 in Alambari (P6), 1 in Capão Bonito (P7), 1 in Guareí (P8), and 2 in Porto Feliz (P9 and P10). The breed of goats were Saneen (P1, P2, P3, P4, P7, P8 and P9), Anglo Nubian (P10) and Alpine (P5, P6 and P7). The samples were collected monthly from May 2010 to September 2011, totalizing 128 samples of feed and 120 samples of milk. In all properties, the feeds are prepared using corn, cottonseed, soy beans and salt and they were stored in barrels, each of them with 20 kg capacity. Feed samples (500 g) were collected from nine different points of each barrel: three points at the upper third, three points at the medium third, and three points at the lower third (Sassahara et al., 2003). The samples were homogenized and stored in plastic containers, and sent to the laboratory for analysis of mycotoxins (aflatoxins and cyclopiazonic acid) and water activity. The bulk milk samples were collected in duplicate and conditioned in sterile 500 mL bottles on the day of the feed collection. The samples were frozen until the time of mycotoxin analysis.

2.2 Aflatoxins Analysis in Feed for Goats

For analysis of aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) in feed, 50 g of each sample, after addition of 5.0 g of NaCl, was transferred to a wide-mouth flask; next, addition of 100 mL of methanol: water (80:20 v/v). After stirring for 30 minutes with a horizontal mechanical stirrer, the flask content was filtered through filter paper, and 10 mL of the extract was diluted in 40 mL of distilled water. Then, 5.0 mL of the filtered extract (5.0 mL = 0.5 g of the sample) was transferred to an immunoaffinity column with a flow rate of ca. 1-2 drops/s until allow the air to pass through the column. After completion of the operation, the column was washed with 10 mL of distilled water with a flow rate of ca. 2 drops/s and eluted twice with 1.0 mL of HPLC grade methanol with a flow rate of ca. 1-2 drops/s. After elution, the extract was collected and stored at freezing temperature until the measurement time.

The high-performance liquid chromatograph (HPLC) used for analysis of mycotoxins was from Shimadzu model LC 6AD, equipped with fluorescence detector RF-551 Shimadzu and ultraviolet (UV) detector - Shimazu SPD-6AV. Aflatoxins were identified and quantified at the following chromatographic conditions: as mobile phase, methanol: acetonitrile: 0.1% phosphoric acid (24:24:52 v/v/v); flow rate of 1.0 mL/min; fluorescence detector (at 364 nm excitation and 440 nm emission), ODS C_{18} column of 4.6 × 250 mm, dia 5 µm (Shimadzu) and oven temperature of 30 ^{o}C (Chan et al., 2004). The quantification method of the samples was via external standard calibration curve using 5 points for each aflatoxin. The points were: 8.25, 11.30, 16.50, 24.75, and 33.0 ng/mL for AFB1; 1.01, 1.4, 2.02, 3.03, and 4.04 ng/mL for AFB2; 2.89, 3.55, 5.79, 8.68 and 11.50 ng/mL for AFG1; and 1.92, 2.67, 3.84, 5.76, 7.68 ng/mL for AFG2. The squared correlation coefficient (r^2) was 0.991 for AFB1, 0.998 for AFB2, 0.964 for AFG1 and 0.999 for AFG2. The quantification limits were established for AFB1, AFB2, AFG1 and AFG2 at 3.5 ng/g, 0.1 ng/g, 2.0 ng/g, and 0.15 ng/g, respectively. The detection limits were 2.0 ng/g, 0.05 ng/g, 1.0 ng/g, and 0.05 ng/g for AFB1, AFB2, AFG1 and AFG2, respectively. The identity

of the aflatoxins was confirmed in accordance with Tarin et al. (2004), where 500 mL of each sample was dried under nitrogen, diluted with 50 mL of trifluoroacetic acid (TFA) and 200 mL of hexane at 40 ^{o}C. The solution was stirred for 15 minutes, dried under nitrogen and redissolved in 200 mL of acetonitrile: water (3:7 v/v) and analyzed by high performance liquid chromatography (HPLC).

2.3 Aflatoxin M1 Analyze in Goat's Milk

Aflatoxin M1 was analyzed in the milk samples according to the methodology described by Dragacci et al. (2001), where 50 mL of each milk sample was centrifuged to remove fat. The resulting skimmed milk was applied to an immunoaffinity column with a flow rate of 1 to 2 drops/s and, afterwards, the column was washed with distilled water. The sample was eluted with 1.25 mL of acetonitrile: methanol (3:2 v/v) and subsequently collected to be washed with 1.25 mL of water and again collected in the same vial. Twenty microliters (20 µL) of the extracted sample was injected into the high performance liquid chromatograph column, with the mobile phase water: acetonitrile: methanol (68:24:8) at a flow rate of 1.0 mL/min and a fluorescence detector at 360 nm excitation and 440 nm emission (Dragacci et al., 2001). For quantification of the samples, a 5 point calibration curve (0.355, 0.71, 0.177, 1.704, and 2.148 µg/mL) was used, and the squared correlation coefficient was 0.995. The quantification and detection limits were 0.09 ng/mL and 0.07 ng/mL, respectively.

2.4 Cyclopiazonic Acid Analysis in Feed for Goats

CPA was analyzed in samples of feed, where 25 g of each sample was transferred to a wide-mouth flask plus 100 mL of methanol: 2% sodium bicarbonate in water (7:3 v/v). After stirring for 30 minutes with a horizontal mechanical stirrer, the flask content was filtered through filter paper. A 50 mL aliquot of extract was transferred to a separatory funnel, followed by addition of 100 mL of hexane. After a gentle stirring, the hexane fraction was discarded, followed by addition of 50 mL of a 10% KCl aqueous solution. This solution was acidified with 2.0 mL of 6 N HCl. CPA was eluted with 50 mL of chloroform twice. After adding anhydrous sodium sulfate, the chloroform fraction was filtered and evaporated to dryness in a rotary evaporator. The residue was resuspended in 10 mL of chloroform and transferred to a solid phase extraction column Micotox® MS2300 (Micotox Ltd., DC Bogota, Colombia) at a flow rate of 1-2 drops/s until allow the air to pass through the column. After completion of the operation, the column was washed with 10 mL of ethyl ether, 10 mL of chloroform: acetone (1:1 v/v) and 10 mL of chloroform: methanol (95:5 v/v). CPA was eluted with 10 mL of chloroform: methanol (75:25 v/v) at a flow rate of 2.0 mL/min. The extract was evaporated under nitrogen to dryness and diluted in 1.0 mL of HPLC grade methanol and stored at freezing temperature until the measurement time.

2.5 Cyclopiazonic Acid Analysis in Goat's Milk

Concerning milk, CPA was extracted using 25 mL of each sample with 25 mL methanol: 2% sodium bicarbonate in water (7:3 v/v) and 100 mL of hexane. After 3 minutes of centrifugation, the hexane fraction was then discarded, and the sample acidified with 2.0 mL of 6 N HCl and transferred to a separatory funnel. CPA was eluted with 100 mL of chloroform twice. After adding anhydrous sodium sulfate, the chloroform fraction was filtered and evaporated to dryness in a rotary evaporator. The residue was taken up in 5.0 mL of chloroform and transferred to a solid phase extraction column Micotox® MS2300 (Micotox Ltd., DC Bogota, Colombia) at a flow rate of about 1-2 drops/s until allow the air to pass through the column. After completion of the operation, the column was washed with 10 mL of ethyl ether, 5.0 mL of chloroform: acetone (1:1 v/v). CPA was eluted with 10 ml of chloroform: methanol (75:25 v/v) at a flow rate of 2.0 mL/min. The extract was evaporated under nitrogen to dryness and diluted in 1.0 mL of HPLC grade methanol. The extract was stored at freezing temperature until measurement time (Prasongsidh et al., 1998).

Cyclopiazonic acid was identified by high performance liquid chromatography using as a mobile phase acetonitrile: ammonium acetate buffer (0.05 M, pH 5) (8:2 v/v), at a flow rate 0.6 mL/min, UV detector 284 nm, ODS C8 column of 4.6 × 250 mm, dia 5.0 µm (Shimadzu). The quantification method of the samples was via external calibration by a 5-points calibration curve (0.765, 1.531, 3.062, 6.125, 12.247 g/mL), and the squared correlation coefficient was 0.995. The limits of detection and quantification were 0.03 µg/g and 0.005 µg/g, respectively.

2.6 Water Activity Determination

The water activity (a_w) of goat feed and forage samples was determined by automatic analysis using Aqualab 4TE (Decagon Devices Inc., Pullman, WA).

2.7 Statistical Analysis

The results were assessed by analysis of variance (ANOVA) with a significance level of $p < 0.05$, and the Tukey-Kramer test with a significance level of $p < 0.05$ and $q > 4.457$ for analysis of variance for multiple

comparisons using the variables: aflatoxins and CPA contamination in feed and their residues in milk. The GraphPad INSTAT 3:10 program was used.

3. Results and Discussion

Management of animal nutrition has great importance on the milk quality and its by-products. Caring with feeds should extend not only to the nutritional value, but also to their quality, because some compounds, such as cottonseed, cottonseed meal and corn are widely used in animal diet in lactation. They usually are ingredients very susceptible to contamination by mycotoxins and toxigenic fungi. Presence of mycotoxins in food for animal consumption was reported in different regions of Brazil (Sassahara et al., 2003; Gonçalez et al., 2004; Oliveira et al., 2008). *A. flavus* capable of producing CPA and aflatoxins were isolated from feed samples studied in this work (Silva et al., 2015).

Of the 128 feed samples analyzed, 2.34% were contaminated with aflatoxins. Aflatoxins were detected in 1 feed sample from P2 property, and in 2 samples from P9 property. Property P2 presented the following aflatoxin concentrations: 36.93 µg/kg AFB1, 2.46 µg/kg AFB2, 46.06 µg/kg AFG1, and 1.65 µg/kg AFG2, and the sum of the four aflatoxins (87.1 µg/kg) exceeded the maximum limit permitted by Brazilian law for feed ingredients (50 µg/kg – total sum of AFB1, AFB2, AFG1, AFG2) (Brazil, 1988). Feed samples from P9 property presented the following aflatoxin concentrations: 3.91 µg/kg AFB1, 1.18 µg/kg AFB2, 3.58 µg/kg AFG1, 0.93 µg/kg AFG2, and 3.65 µg/kg AFB1; 0.35 µg/kg AFB2, 0.61 µg/kg AFG2, and AFG1 was only detected (Table 1). Batatinha et al. (2008) reported the absence of aflatoxin in 80 feed samples for goats in Brazil.

Concerning CPA, of the 128 samples of feed analyzed, 3.12% were contaminated. This was detected only in samples from P1 and P3 properties, but its quantification was not possible since the values were below the quantification limit (3.0×10^{-5} µg/kg). In samples from P8 and P9 properties one found 0.743 µg/ g and 1.9 µg/kg of CPA, respectively (Table 1). Brazil does not have the maximum limit permitted for CPA in feed.

The low level of aflatoxin contamination and CPA in feed samples for feeding goats in lactation may be related to sample water activity indices ranging from 0.51 to 0.75, values below those required for growth of *A. flavus* and *A. parasiticus* (0.78 to 0.80) as well as for the production of aflatoxins (0.83-0.87) and CPA (0.94). All properties stored their feeds for a period not exceeding 15 days and in protected locals, free of moisture and rodents, factors that also contributed to the low infection rate of both contaminants.

Table 1. Concentration of aflatoxins (µg/kg) and CPA (µg/kg) in feed samples for dairy goats collected from familiar properties in the Southwestern São Paulo region

Properties	Total of samples analyzed (positive)	Mycotoxins (µg/kg)					
		AFB1	AFB2	AFG1	AFG2	Total AFs	CPA
P1	17 (0)	ND	ND	ND	ND	-	D
P2	3 (1)	36.93	2.46	46.06	1.65	87.1*	ND
P3	8 (0)	ND	ND	ND	ND	-	D
P4	14 (0)	ND	ND	ND	ND	-	ND
P5	9 (0)	ND	ND	ND	ND	-	ND
P6	19 (0)	ND	ND	ND	ND	-	D
P7	16 (0)	ND	ND	ND	ND	-	ND
P8	7 (0)	ND	ND	ND	ND	-	0.743
P9	18 (2)	3.91	1.18	3.58	0.93	9.6	ND
		3.65	0.35	1.14	0.61	5.75	1.9
P10	17 (0)	ND	ND	ND	ND	-	ND

Note. ND: not detected; D: detected; AFB1: aflatoxin B1; AFB2: aflatoxin B2; AFG1: aflatoxin G1; AFG2: aflatoxin G2; Total AFs: aflatoxins (B1+B2+G1+G2); CPA: cyclopiazonic acid; *Concentration beyond the maximum limit permitted by Brazilian law.

Aflatoxin M1 and CPA were not found in the 120 milk samples analyzed. The absence of AFM1 and CPA evaluated in all milk samples was positively correlated with the results of the analyses of feed and their statistical significance ($p < 0.05$), which exhibited a low rate of occurrence of AFB1 and CPA in animal nutrition for the same experimental period. Although three feed samples positive for aflatoxin B1 and four positive for CPA have

been encountered AFM1 and CPA were not detected in their respective milk. The absence of AFM1 may be related to the rate of elimination from 0.18% to 3.0% of ingested AFB1. Studies on the elimination of CPA are scanty. Dorner et al. (1994) presented a study in sheep, where from each 5 μg/kg body weight, 236 ng of CPA/g was quantified by HPLC on the first day, hence showing a very low rate of the toxin elimination. Negative results for the presence of AFM1 in milk and cheese of goat were also reported by other authors (Polat et al., 1990; Botura, 2005; Tsiplakou et al., 2014.). In Brazil, AFM1 in goat milk was detected by Barrios et al. (1996), and Oliveira and Ferraz (2007), who observed that 45% and 69.4% of the samples contained AFM1 at levels ranging from 20-200 μg/kg and 0.011 to 0.161 ppb, respectively. In Italy, AFM1 analysis in samples of milk and cheese of goat showed that about 30% of the samples were contaminated by this toxin at levels from 0.004 to 0.037 μg/kg in milk and from 0.019 to 0.16 μg/kg in cheese (Finoli & Vecchio, 1997). A study carried out in Sardinia in 208 goat milk samples and 41 goat cheese samples detected the presence of AFM1 in 36 (17.3%) at a concentration from 5.0 to 40 ng/L of milk and 9.8% at levels between 79.5 and 389 ng/kg of cheese, respectively (Virdis et al., 2008).

4. Conclusion

The results of the study showed low contamination of feed by aflatoxins and CPA and, consequently, their absence in milk. On despite this, it is very important periodically monitoring the level of mycotoxins in the feed for dairy goats to prevent the milk contamination, once this food is consumed by human, specially children.

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