

Determination of fungal microbiota and mycotoxins in brewers grain used in dairy cattle feeding in the State of Bahia, Brazil

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Abstract

The aim of this study was to determine the mycoflora and evaluate the presence of aflatoxins and ochratoxins in brewers grain used to feed dairy cattle in the State of Bahia. Twenty samples of brewers grain were collected each trimester, during a whole year, in five properties located in cities of the “recôncavo baiano” (Bahia, Brazil) for a total of 80 samples. Samples were analyzed for aflatoxins and ochratoxins by fluorimetry with immunoaffinity columns. *Aspergillus* was the most frequently isolated genus (42.5%), followed by *Penicillium*, *Mucor*, *Rhizopus* and *Fusarium*. Mycotoxicological analyses did not show the presence of ochratoxins, but the presence of aflatoxins was observed in 33.75% (27/80) of the samples, with contamination levels between 1 and 3 µg/kg.

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1. Introduction

The dairy cattle herd of the State of Bahia consists of 1,508,904 heads and produces 752,026,000 l of milk/year, corresponding to 3.5% of the national production (IBGE, 2003). The expansion of intensive and semi-intensive systems for the rearing of these animals has increased the need for storage of feed used during prolonged dry periods. The use of agroindustrial residues as a food supplement for dairy cattle plays a significant economic role due to the availability and versatility of these materials. Brewers grain are one of the most frequently used by-products mainly because of the proximity between breweries and dairy

properties in this region, which reduces the cost of transportation of this material.

The microregions in which these dairy herds are reared are characterized by high temperatures, reaching up to 38 °C, a mean annual rainfall above 1000 mm and relative air humidity greater than 70% (Anuário, 2002). These climatic conditions, together with the inadequate storage of brewers grain in the farms and the composition of this substrate, provide the ideal conditions for fungal development.

Various fungi produce mycotoxins, chemically diverse secondary metabolites that can be harmful both to human and animal health (CAST, 2003; Fink-Gremmels, 1999). Evidence shows the participation of these toxic agents in the etiology of serious pathological processes in production animals associated with animal feeds, including barley, and this kind of contamination has been the subject of intensive scientific investigation both in Brazil and in different parts

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of the world (Kim, Kang, Lee, Lee, & Yoshizawa, 1993; Mallmann, Santurio, & Dilkin, 1999). Aflatoxins and ochratoxins are among the various mycotoxins identified that play an important role in animal health.

Because of the marked risk for animal health and productivity due to exposure to mycotoxin, continuous studies regarding the monitoring of these toxins in products to be used as animal feed are being performed worldwide.

In Brazil, most reports on the contamination of food products by mycotoxins refer to the south and southeast regions of the country, and mainly indicate the presence of aflatoxins, fumonisins, zearalenone, ochratoxin A and deoxynivalenol (Rodriguez-Amaya & Sabino, 2002). In view of the scarcity of information regarding the occurrence of mycotoxins in animal feed in the State of Bahia, the aim of the present study was to investigate fungal microbiota and the presence of aflatoxins and ochratoxins in brewers grain used as dairy cattle feed in this state.

2. Material and methods

2.1. Sample collection

Brewers grain samples were collected in five farms (A, B, C, D and E) located in the cities of the “recôncavo baiano” (exceptionally fertile region on the coast of the State of Bahia, Brazil). These properties were selected because they have the largest concentration of dairy cattle and constantly use this material as animal feed. In each property, four samples were collected every three months over a period of one year, in a total of 80 samples. The mean period the product remained in the storage tanks was considered the determining factor for sample collection.

A questionnaire regarding sanitary and nutritional management of the herd, as well as storage conditions and form of use of brewers grain, was applied during the visits to the farms.

2.2. Determination of the moisture content of the samples

The moisture content of brewers grain samples was determined according to the method by Silva (1991). Samples were previously weighed and dried at 105 °C until they reached a constant weight. Moisture content of each sample was determined by the difference between dry weight and initial weight.

2.3. Isolation of mycoflora (Swanson, Busta, Peterson, & Johnson, 1992)

Ten grams of each brewers grain sample was diluted in 90 ml sterile distilled water to obtain a 10^{-1} dilution. Using this stock solution, serial dilutions up to 10^{-4} were then prepared with the same diluent. Aliquots of 1 ml of each dilution were plated on Petri dishes containing 15 ml potato dextrose agar supplemented with 100 µg/ml chloramphenicol, and the plates were incubated at 25 °C for 5

days. Filamentous fungi were identified at the genus level (Nelson, Tousoun, & Marasas, 1983; Raper & Fennell, 1965).

2.4. Determination of mycotoxins in brewers grain

2.4.1. Total aflatoxins (Aflatest Manual, 2002)

Fifty grams of each sample were homogenized in a blender with 100 ml methanol:water (80:20) and 5 g NaCl for 1 min. After filtration, a 10-ml aliquot was completed to a volume of 50 ml with distilled water. The sample was then filtered a second time using microfiber paper and 10 ml of this filtrate was applied to an immunoaffinity column (Aflatest[®], Vicam). After passage of the sample, the column was washed twice with 10 ml distilled water and then with 1 ml methanol to elute aflatoxins bound to monoclonal antibodies. The Aflatest[®] agent used in the development was added to the eluate and aflatoxin content was read with a fluorimeter (series 4, Vicam). Detection limit of the method was 1 µg/kg.

2.4.2. Ochratoxins (Ochratest Manual, 2002)

For analysis of ochratoxins, 50 g of each sample were homogenized in a blender with 100 ml methanol:water (80:20) for one minute. After filtration, a 10-ml aliquot was completed to a volume of 50 ml with distilled water. This solution was then filtered a second time using microfiber paper and 10 ml of this filtrate was applied to an immunoaffinity column (Ochratest[®], Vicam). After passage of the sample, the column was washed with 10 ml ochratoxin buffer solution and 10 ml distilled water. After that, 1.5 ml Ochratest[®] solution was used to elute ochratoxin bound to monoclonal antibodies. This eluate was immediately read using a fluorimeter (series 4, Vicam). Detection limit of the method was 2 µg/kg.

2.4.3. Quality control of the analyses

Tests were performed in triplicate to validate the protocols used in this study. In order to do this, confirmed negative samples were contaminated with a known amount of each mycotoxin standard (Sigma). The confirmation of negative samples were obtained through HPLC method at the Lamic (Laboratory of Mycotoxins, University of Santa Maria, Brazil) (Mallmann et al., 2000). After extraction, percentile of recovery was calculated as the difference between the fluorimetric value obtained for the test sample and the initial contamination of the same sample.

Recovery was 89.58% (cv% = 8.97) and 66.66% (cv% = 35.34) for aflatoxins and ochratoxin A, respectively.

2.5. Climatological data

Climate information regarding mean, minimum and maximum temperature, mean rainfall and relative air humidity on the properties studied was provided by the National Institute of Meteorology of the State of Bahia (INMET-BA). The period of storage of brewers grain in the

farms was the determining factor in the calculation of the mean climatological data used here.

2.6. Statistical analysis

The influence of climatological factors on the presence of fungi and mycotoxins in brewers grain was determined by univariate analysis of variance, followed by the Student *t*-test for the analysis of differences between means of the groups, using the SPSS statistical software. In addition, the correlation between the presence of fungi and mycotoxins was determined using Pearson's correlation coefficient.

3. Results and discussion

Analysis of the information obtained from the questionnaires applied to the farms studied revealed a semi-intensive animal rearing system with a closely similar feeding regimen consisting of bulky feed and brewers grain, although other agroindustrial residues such as sugarcane were also added to the diet, when available. Brewers grain were offered at a proportion of 15–20 kg/animal/day to lactating cows; however, in two farms, all cattle received the product. Brewers grain were stored in cement tanks with a capacity of approximately 20 tons in all properties, except for farm C, where this material was kept on a cemented surface covered with a black plastic sheet. The storage period varied as a function of the number of lactating cows, generally ranging from 8 to 15 days. As a conservation measure, common salt was added to the brewers grain as it arrived in the farms.

Analysis of the climatic factors demonstrated no significant difference in minimum or mean temperature between properties, while the maximum temperature in farm E was lower than in the other farms (Table 1). The highest temperatures were observed during the 4th collection period (Table 2).

Rainfall levels and relative humidity were significantly higher during the 1st and 2nd collection periods. The moisture content of the samples also showed significant variation, with the lowest value being observed in farm C, possibly due to the form of storage of brewers grain in this property (Table 1).

Analysis of mycoflora of the 80 brewers grain samples revealed *Aspergillus* spp. as the most frequent (42.5%) genus isolated, followed by *Mucor* spp. (32.5%), *Rhizopus* spp. (32.5%), *Penicillium* spp. (7.5%), and *Fusarium* spp. (2.5%). *Cladosporium* spp. was detected in only one sample.

The predominance of fungi of the genus *Aspergillus*, together with the reduced presence of *Fusarium* observed for all samples studied (Table 2) may be the result of brewers grain storage and processing conditions. These conditions may have favored the development of storage and contaminant fungi instead of those known as field fungi, which include the genus *Fusarium*, more frequently found on recently harvested grains than on processed and stored ones (Lillehoj, 1973). Abramson, Hulasare, White, Jayas, and Marquardt (1999) and Hill and Lacey (1983) also found a higher frequency of *Aspergillus* and *Penicillium* when analyzing stored barley grains.

Ackermann (1998) and Lee et al. (1986), who analyzed malted barley grains, observed the presence of the genera

Table 1
Mean climatic conditions in the properties and during each sample collection

Climatic conditions	Property					Collection			
	A	B	C	D	E	1 ^a	2 ^a	3 ^a	4 ^a
Min. temperature (°C)	20.9	20.9	20.9	20.4	19.9	20.4 ^A	19.4 ^B	20.3 ^A	22.9 ^C
Mean temperature (°C)	25.1	25.1	25.1	24.8	23.9	24.9 ^A	22.5 ^B	25 ^A	27.8 ^C
Max. temperature (°C)	30.9 ^a	30.9 ^a	30.8 ^a	30.9 ^a	28.5 ^b	30.5 ^A	27 ^B	31.3 ^A	34.7 ^C
Mean rainfall (mm ³)	40.9 ^a	40.9 ^a	59.8 ^{a,b}	79.5 ^{b,c}	230.4 ^c	136.6 ^A	93.4 ^B	5.6 ^C	31.6 ^{B,C}
Relative air humidity (%)	75 ^a	75 ^a	75.8 ^{a,b}	76.8 ^{a,b}	83.5 ^b	80.4 ^A	87.6 ^B	70.8 ^C	63.5 ^D
Moisture content (%)	73.3 ^a	74.6 ^a	69.3 ^b	75.4 ^a	74.5 ^a	75.3 ^A	73.6 ^{A,B}	71.8 ^B	72.9 ^{A,B}

Note: Uppercase shows the comparison of values in the rows between properties, and lowercase shows the comparison of values in the collection of samples ($p < 0.05$).

Table 2
Mean fungal contamination in the properties and during each sample collection ($\times 10^3$ CFU/g)

Fungi	Property					Collection			
	A	B	C	D	E	1 ^a	2 ^a	3 ^a	4 ^a
<i>Aspergillus</i>	2.96	0.11	1.05	0.17	0.41	0.42	0.72	2.5	0.12
<i>Penicillium</i>	0	0	0	0	0	0.11	0	0	0
<i>Fusarium</i>	0	0	0	0	0.63	0	0	0.51	0
<i>Mucor</i>	0.5	0.42	0	0.6	0.53	0.58	0.78	0.13	0.15
<i>Rhizopus</i>	0	0.78	0.17	0.73	1.21	0.83	0.36	1.01	0.11
<i>Cladosporium</i>	0	0	0	0	0	0	0	0	0
Total	3.46	1.31	1.22	1.5	2.78	1.94	1.86	4.15	0.38

Note: Uppercase shows the comparison of values in the rows between properties, and lowercase shows the comparison of values in the collection of samples ($p < 0.05$).

Alternaria, *Aspergillus*, *Fusarium*, *Mucor*, *Penicillium*, *Cladosporium*, and *Rhizopus*. However, the most frequent fungi found on barley grains collected in the field were *Alternaria*, *Fusarium* and *Cladosporium*; *Aspergillus* and *Penicillium* were also detected, but in smaller amounts (Ackermann, 1998; Sanchis, Sanclemente, Usall, & Viñas, 1993).

The greatest frequency of fungi was observed during the 3rd collection period (Table 2). The temperature and moisture content of the samples may have influenced these results since the lowest rainfall was observed during this period (Table 1). According to Hill and Lacey (1983), moisture content greater than 40% shows water activity equal to 1, an index favorable to fungal development. The high moisture content of the samples analyzed is the result of conservation of brewers grain in water. Rainfall played no role in this measurement, although this factor usually interferes with air humidity and consequently with the moisture content of the sample. Samples from farm C showed the lowest level of fungal contamination (Table 2), possibly because of the management of brewers grain in this property. The product was consumed within a maximum period of 5 days and stored in such a way that the existing water could be drained, enabling the drying of the barley.

High moisture content of substrates stored for long periods provides adequate microclimate for fungal development and, consequently, mycotoxin production (Sundlof & Strickland, 1986). Fungi of the genus *Fusarium* generally invade and colonize grains with moisture content between 22% and 25%, while *Aspergillus* and *Penicillium* are found in grains with moisture content between 13% and 18% (Lillehoj, 1973).

Aspergillus spp. were most frequently detected in farm A during the 3rd collection, characterized by mean temperature of 25 °C and 70–75% relative humidity. Fungi of the

genus *Penicillium* were observed during the 1st collection period, when climatic conditions were ideal for fungal proliferation. The genus *Fusarium* was detected during the 3rd collection period, when temperature and humidity conditions were adequate despite a low rainfall (Table 2).

The genus *Cladosporium* was only detected in one sample (0.15×10^3 CFU/g) obtained during the 1st collection in farm E. The genera *Mucor* and *Rhizopus* were most frequently found in samples derived from the 2nd and 3rd collections, respectively.

In the present study, no correlation was observed between climatic factors and the frequency of *Aspergillus* and *Penicillium*, although Silva, Pozzi, Mallozzi, Ortega, and Correa (2000) reported some of these climatic factors, such as maximum and mean temperature, rainfall and grain water content, to be determining factors in the development of these genera.

A positive correlation was observed between the genus *Fusarium*, temperature (mean, minimum and maximum) and air humidity. Such correlation was also observed by Pozzi et al. (1995) who investigated the presence of fungi in corn grains. Other factors, such as rainfall and relative humidity, may also influence the development of this fungus (Silva et al., 2000). Mean rainfall also correlated with the development of *Mucor*, *Rhizopus* and *Cladosporium* in samples analyzed in the present study.

Analysis of mycotoxins in brewers grain revealed contamination with aflatoxins, while no ochratoxins were detected. Twenty-seven (33.75%) of the 80 samples analyzed were positive for aflatoxins ($B_1 + B_2 + G_1 + G_2$), with levels ranging from 1 to 3 µg/kg (Tables 3 and 4).

The highest aflatoxin concentrations were observed in samples derived from farm B and the lowest levels were detected in farm D, although the difference was not

Table 3
Number of samples positive for the presence of aflatoxins ($B_1 + B_2 + G_1 + G_2$) collected from March 2002 to May 2003

Property	Collection				Total of positive samples (%)
	1st	2nd	3rd	4th	
A	0	0	2	3	5 (31.25)
B	1	2	4	1	8 (50)
C	1	0	1	1	3 (18.75)
D	1	0	2	1	4 (25)
E	0	1	2	4	7 (43.75)
Total of positive samples	3 (15)	3 (15)	11 (55)	10 (50)	27 (33.75)

Table 4
Mean concentration of total aflatoxins (µg/kg) in barley samples collected from March 2002 to May 2003

Property	Collection				Mean
	1	2	3	4	
A	0 ^a	0 ^a	1.25 ^b	0.75 ^{a,b}	0.5 ^A
B	0.25 ^a	0.75 ^{a,b}	1.5 ^b	0.5 ^{a,b}	0.75 ^A
C	0.75 ^a	0 ^a	0.5 ^a	0.25 ^a	0.38 ^A
D	0.25 ^a	0 ^a	0.5 ^a	0.5 ^a	0.31 ^A
E	0 ^a	0.25 ^a	0.5 ^a	1.25 ^b	0.5 ^A
Mean	0.25 ^a	0.2 ^b	0.85 ^c	0.65 ^{a,c}	–

Note: Uppercase shows the comparison of values in the rows, and lowercase shows the comparison of values in the columns ($p < 0.05$).

significant. As for the collection period, the highest levels were detected in brewers grain samples obtained during the 3rd period (Table 4).

No correlation could be established between the presence of fungi and toxin levels in the samples. However, a positive correlation was observed between aflatoxin level, mean rainfall and relative humidity, factors that have an expressive influence on moisture content of the samples. The lack of a correlation between aflatoxin levels and fungal development/climatic conditions observed in this study suggests that the production of these toxins may have occurred before storage of the brewers grain in the farms, or that the *Aspergillus* strains detected are able to produce large amounts of toxins.

Scientific reports on the contamination of animal feed with mycotoxins in the State of Bahia are scarce. Only Batatinha et al. (2003) and Bautista et al. (1989) reported the presence of aflatoxins in corn and peanuts, and their by-products, with an incidence of 12% and 58%, respectively.

In the present study, aflatoxin concentrations ($B_1 + B_2 + G_1 + G_2$) found in brewers grain were below the levels allowed for animal feed by legal regulations (Decreto MA/SNAD/SFA no. 7 from November 9, 1988), which establish a maximum level of 50 µg/kg for any raw material to be used directly or as an ingredient in feed destined for animal consumption. However, the presence of this mycotoxin in this substrate indicates the existence of contamination, a fact that would require periodic monitoring because of the favorable climatic conditions for fungal development and the inadequate form of storage that favors the proliferation of *Aspergillus flavus* and *Aspergillus parasiticus*, the main producers of these toxins. This is the way by which the occurrence of aflatoxicosis in production animals could be prevented, minimizing economic losses and reducing the risk posed by the transmission of highly carcinogenic metabolites (aflatoxin M_1) into milk, both for human and animal health.

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