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Fungal and mycotoxins contamination in corn silage: Monitoring risk before and after fermentation

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ABSTRACT

Silage is a widespread practice to preserve forage. Poor storage conditions can lead to mold contamination and mycotoxin production. The aim of this study was to establish the occurrence of toxigenic fungal species and to determine aflatoxins (AFs), ochratoxin A (OTA), fumonisin B_1 (FB_1) and deoxinivalenol (DON) in corn silage intended for bovines before and after fermentation in farms located in São Paulo and Rio de Janeiro States, Brazil. Fungal counts were done by surface-spread method. Toxigenic ability of isolates was evaluated *in vitro*. AFs natural contamination was determined by TLC and HPLC. Total fungal counts were generally high. *Aspergillus flavus*, *Penicillium citrinum*, and *Fusarium verticillioides* were the prevalent species. Toxigenic strains were isolated. Aflatoxin levels differed ($P < 0.0001$) from 2 to 45 μg g^{-1} and from 2 to 100 μg g^{-1} in pre and post-fermentation samples, respectively. Ochratoxin A, FB_1 and DON levels found in pre-fermentation samples were higher than in post-fermentation ($P < 0.0001$). Mycotoxins and toxigenic fungi were present before and after fermentation in corn silage intended for bovines in Brazil. Aflatoxin B_1 (AFB_1) increased during storage whereas OTA, FB_1 and DON decreased. Determination of mycotoxin levels and their occurrence in corn silage are important to provide information so that the assessments of risk for animal feed and livestock environment could be made.

1. Introduction

Corn (*Zea mays* L.) is the most widely grown crop in the Americas extensively used for animal feeding and human consumption due to its nutritional value. Silage is a widespread practice to preserve forage during extended time periods. The production of corn silage entails incorporation of the whole plant and its storage is based on the principle of preservation under anaerobic conditions with the growth of lactic acid bacteria. These bacteria promote a natural fermentation that lowers the pH to a level that is considered unfavorable for the growth of clostridia and most molds (Richard et al., 2007). Temperature, humidity, oxygen availability and pH conditions vary during the silage process and the microbiota may also change from one stage to another. Nutritional value of silage is similar to that of fresh forage. However, poor storage conditions such as excessive moisture or dryness, condensation, heating, leakage of rainwater and insect infestation can lead to undesirable mold contamination, mycotoxin production and the reduction of nutritional value (Dos Santos et al., 2003; Boysen et al., 2000; Cleveland et al., 2003; Frisvad et al., 2006; Nout et al., 1993; Richard et al., 2009). Mycotoxins are low molecular weight products of the fungal secondary metabolism, produced mainly by *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* genera. These metabolites are toxic to humans and animals when consumed or inhaled, and exposure to mycotoxins through contaminated feed is one of the major risks

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affecting ruminant health (Bennett and Klich, 2003; Kalac and Woolford, 1982). Toxic syndromes caused by mycotoxin ingestion are indicated as mycotoxicosis and their toxic effects are diverse depending on the toxin. Aflatoxins are potent carcinogenic toxins. Ingestion of hepatotoxic aflatoxin B_1 (AFB_1) by lactating animals can induce the presence of aflatoxin M_1 in milk (Corbett et al., 1988). Acute aflatoxicosis in cattle has been also thoroughly described (Bodine and Mertens, 1983; CAST, 2003). Other mycotoxins such as trichothecenes, OTA and zearalenone alter immune-mediated activities in bovines (Black et al., 1992). Fumonisins have been associated with the occurrence of pulmonary edema in pigs, leukoencephalomalacia in horses, hepatic cancer in rats and esophageal cancer in humans (Howard et al., 2001; Smith et al., 2002; Marasas et al., 2000; Marasas, 2001). In contrast, cattle appear to be quite resistant to FBs due to its limited absorption and metabolism (Osweiler et al., 1993; Rice and Ross, 1994). Surveillance for mycotoxins in cereals and animal feeds has shown that more than one toxin can occur in the same commodity (Scudamore et al., 1998). In Brazil, about 89% of the total corn production is destined to animal feeding (Rodrigues et al., 2002). The use of silage to preserve feed is a relatively new practice in our region. The environmental conditions in São Paulo and Rio de Janeiro are different from other parts of the world where data on mycological and mycotoxin contamination in silage have been reported. At the present time, there is no available data on exposure levels of *Aspergillus* and *Fusarium* mycotoxins from silage in Brazil. The prevalent environmental conditions, together with inadequate feed storage provide suitable conditions for fungal development. Most reports on the contamination of corn silage by fungi and mycotoxins in Latin America refer to other countries (González Pereyra et al., 2008; Reyes-Velázquez et al., 2008). In view of the scarcity of information the aim of the present study was to establish the natural occurrence of toxigenic fungal species and to determine the presence of AFs, OTA, FB_1 and DON in corn silage samples destined to bovine consumption before and after the fermentation process in the silo (pre and post-fermentation).

2. Materials and methods

2.1. Sample collection

The samples were collected in one of the most important regions of Brazil where silage practice is developed. A total of 464 samples of corn silage were collected from 58 different silos during two sampling periods (232 in each sampling), from June 2007 to October 2007 and from February 2008 to May 2008. These silos were placed in two farms located São Paulo and two located in the State of Rio de Janeiro. The silos were representative in size, composition and kind of storage method applied. Each silo was sampled at two different times: before compaction (pre-fermentation) and after compaction and 90 days of fermentation (post-fermentation). The sampling was performed by collecting material from four different sections of each silo, the upper layer (U), the lower layer (L), the laterals (LT) and the central part (C). A 2 kg sample of silage material was taken from each section. It was homogenized and quartered manually so that a 500 g sample from each sampling site was obtained. All samples were immediately taken to the laboratory, ground in a mill (particle size: 1 mm) and tested for dry matter content (DM%), pH and water activity (a_W). A 10 g aliquot from each sample was randomly selected for the analysis of the mycobiota and the rest was stored at 4 °C until mycotoxin analysis.

2.2. Physical evaluation of the samples

Dry matter percentage (DM%) and pH of the samples were evaluated according to methodology described by Ohyama et al. (1975). Water activity (a_W) was determined using an AQUALAB CX2 (Decagon, Devices, Inc. USA) appliance.

2.3. Mycological survey

Total fungal counts of samples were performed on three different culture media: dichloran rose bengal chloramphenicol agar (DRBC), a general medium used for estimating total culturable mycobiota (Pitt and Hocking, 1997); dichloran 18% glycerol agar (DG18), a low a_W medium that favors xerophilic fungi development (Pitt and Hocking, 1997); and Nash & Snyder agar (NS), a selective medium for *Fusarium* spp. counts (Nelson et al., 1983). 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 duplicate on 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 15–150 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 Klich (2002), Nelson et al. (1983), Samson et al. (2000), and Samson and Frisvad (2004). The results were expressed as isolation frequency of the fungal genera (% of samples in which each genera was present) and relative abundance of each fungal species (% of isolation of each species among strains of the same genera).

2.4. Toxigenic profile of fungal isolates

The ability to produce OTA by potentially producer strains isolated from corn silage samples (*Aspergillus niger* aggregate) was tested according to the methodology proposed by Téren et al. (1996). Aflatoxins production ability was evaluated in all *Aspergillus* section Flavi isolates according to the methodology described by Geisen (1996).

2.5. Mycotoxin analyses

The natural incidence of seven mycotoxins was evaluated in the 58 corn silos. The presence of aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2) and OTA was evaluated using the extraction methodology described by Soares and Rodeigues-Amaya (1989) combined with an immunoafinity cleanup and a high performance liquid chromatography (HPLC) detection. Briefly, 50 g of each sample were extracted with 270 mL methanol and 30 mL of 4% sodium chloride and homogenized for 30 min in an orbital shaker. One hundred fifty mL of the extracts were filtered through Whatman N° 2 filter paper and 150 mL of 40% ammonium sulfate and 15 g celite were added to the filtrates. The mixture was shaken for 5 min and filtered for a second time as described before. One hundred mL of the clarified extract were collected and transferred to a separation funnel together with 150 mL distilled water and extracted twice with 20 mL chloroform each time. Twenty milliliter of the chloroformed extract were collected and evaporated in a rotary evaporator at 70 °C. The extracts were re-dissolved in 1 mL chloroform and the clean-up was done using Beacon Aflatoxin Immunoafinity Columns (Cat.#20-0097) (Beacon Analytical Systems Inc., Portland, Maine, USA) passing the extract through the column at a rate of 1 drop/s. After that, 2 mL purified water were passed through the column and aflatoxins were eluted with 1 mL methanol. Extracts were collected