Journal of Stored Products Research 52 (2013) 42–47

Contents lists available at SciVerse ScienceDirect

Journal of Stored Products Research

journal homepage: www.elsevier.com/locate/jspr

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

L.A.M. Keller [a,b], M.L. González Pereyra [c,d], K.M. Keller [a,b], V.A. Alonso [c,d], A.A. Oliveira [a,b], T.X. Almeida [a,b], T.S. Barbosa [a], L.M.T. Nunes [a], L.R. Cavaglieri [c,e,*], C.A.R. Rosa [a,f]

[a] Departamento de Microbiologia e Imunologia Veterinária, Universidade Federal Rural do Rio de Janeiro, Instituto de Veterinária, Rodovia BR 465 Km. 7, Seropédica, Rio de Janeiro 23890-000, Brazil
[b] Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Av. Antonio Carlos, 6627 Belo Horizonte, MG 31270-901, Brazil
[c] Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta 36 Km. 601, 5800 Río Cuarto, Córdoba, Argentina
[d] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Rivadavia 1917, C1033AAV Buenos Aires, Argentina
[e] Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Rivadavia 1917, C1033AAV Buenos Aires, Argentina
[f] Conselho Nacional de Pesquisas Científicas (CNPq), Av. Antonio Carlos, 6627 Belo Horizonte, MG 31270-901, Brazil

ARTICLE INFO

Article history:
Accepted 17 September 2012

Keywords:
Aflatoxin B_1
Corn silage
Deoxinivalenol
Fumonisin B_1
Fungi
Ochratoxin A

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

* Corresponding author. Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta 36 Km. 601, 5800 Río Cuarto, Córdoba, Argentina. Tel.: +54 358 467 6113; fax: +54 358 467 6231.
E-mail addresses: lcavaglieri@arnet.com.ar, lcavaglieri@exa.unrc.edu.ar (L.R. Cavaglieri).

0022-474X/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jspr.2012.09.001

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

and evaporated under N_2 flow re-dissolved in 100 μl chloroform and analyzed by HPLC. The HPLC system consisted in a Waters apparatus (Waters Associates™, Inc., Miliford, M.A. – EUA) connected to two Waters (model 510) pumps, a Rheodyne injector (Rheodyne™, Cotati, California, USA) with a 20 mL loop, a Merck-Hitachi UV–VIS L-4250 detector (350 nm wavelength) and a Merck Hitachi D-2500 integrador. A silica column (150 × 4.6 mm, 5 μ) (VARIAN™, Wallnut Crek, CA, USA) was used for separation. The mobile phase used for normal phase HPLC was ethyl acetate:n-hexane (3:2,5 – v/v) at a 1.5 mL min^{-1} flow rate. Aflatoxin (AFB_1, AFB_2, AFG_1, AFG_2) and OTA standard solutions (Sigma Co, St. Louis – EUA) were prepared and calibrated according to AOAC (2000) and quantified in a Shimadzu model 2001 (Shimadzu Co.®, Kyoto, Japan) spectrophotometer. The standard solutions were kept at −18 °C in caramel vials until used. Confirmation tests were done by co-injection and UV spectroscopic analysis of the peak with a Merck-Hitachi L-3000 diode array detector (Hitachi Ltd.™, Japan) and a workstation (D-6 software). Recovery assays of this method were of 80%. The limit of detection for aflatoxins and OTA was ≤0.1 μg g^{-1}.

For DON and FB_1 quantitative analyses, commercial indirect competitive ELISA kits were used (Beacon Analytical Systems Inc., Portland, Maine – USA) following the protocol provided by the manufacturer.

2.6. Statistical analyses

Data analyses were performed by analysis of variance (ANOVA). Data were transformed ($\log_{10}(x + 1)$) before applying ANOVA. Colony forming units data were transformed ($\log_{10}(x + 1)$) before applying the analysis of variance. Duncan's test was used for comparing CFU g^{-1} total fungal counts on different culture media and Fisher's protected LSD test was used for comparing means of mycotoxins contamination and physical evaluation of samples data (Quinn and Keough, 2002). The analyses were conducted using PROC GLM in SAS (SAS Institute, Cary, NC, USA).

3. Results

3.1. Physical evaluation of the samples

Mean DM% of corn silage samples was 39.75% varying in a range from 38 to 42 %. pH values varied from 4.2 to 6.5 with a mean value of 4.88 while a_W varied from 0.924 to 0.992 showing a mean value of 0.960. No statistically significant differences were observed when comparing physical characteristics of pre and post-fermentation samples.

3.2. Mycological survey

Table 1 describes total fungal counts on DRBC and DG18 media encountered in pre and post-fermentation corn silage samples. Fungal counts were significantly higher ($F_{1,5} = 58.01$, $P < 0.001$) in post-fermentation samples. Silage section did not influence the fungal counts ($F_{3,11} = 1.66$; $P < 0.1734$). *Fusarium* spp. counts on NS medium were generally low varying from 1.0×10^2 to 2.0×10^2 CFU g^{-1} in both pre- and post-fermented samples (data not shown).

Seven filamentous fungi genera were isolated from pre and post-fermented silage samples, being *Aspergillus* spp. the most frequent. Yeasts were also present in nine percent of pre-fermented and seven percent of post-fermented samples. Fig. 1 shows the relative abundance values for each of the isolated genera.

Six different *Aspergillus* species and the *A. niger* aggregate were identified. The predominant species identified in pre-fermented samples were *Aspergillus flavus* (40%) and the *A. niger* aggregate (23%), whereas in post-fermentation samples *Aspergillus fumigatus* (17%) abundance was also significant along with the previously mentioned (Fig. 2a).

Penicillium species isolated from pre-fermentation silage samples were *Penicillium citrinum* (65%), *Penicillium islandicum* (25%) and *Penicillium glabrum* (10%). In post-fermentation samples, five species were identified: *P. citrinum* (50%), *P. islandicum* (17%), *Penicillium griseofulvum* (15%), *Penicillium funiculosum* (13%), and *P. glabrum* (5%) (Fig. 2b).

Eighty percent of *Fusarium* spp. strains isolated from pre-fermented silage samples were identified as *F*usarium verticillioides and 20% as *Fusarium graminearum*. All *Fusarium* spp. strains isolated from post-fermented silage samples were identified as *F. verticillioides* (Fig. 2c).

3.3. Toxigenic profile of fungal isolates

Twenty-three percent of *A. niger* aggregate isolates showed ability to produce 2 to 10 μg g^{-1} OTA. Sixty percent of *A. flavus* strains were able to produce 1–5 μg g^{-1} AFB_1 and AFB_2. Similar levels of AFs were produced by 75% of *Aspergillus parasiticus* strains.

3.4. Mycotoxin analyses

Aflatoxin B_1, OTA, DON and FB_1 contamination was detected in corn silage pre as well as post-fermentation and in every sampled section of the silos (U, L, C and LT). Aflatoxin B_2, G_1 and G_2 were not detected in any of the samples. Aflatoxin B_1, DON and FB_1 levels

Table 1
Total fungal counts (CFU g^{-1}) found in corn silage samples collected from different sections of the silo before and after fermentation evaluated on DRBC and DG18 media.

Average total fungal counts (CFU g^{-1}) ± SD in corn silage samples	Section of the silo	Average total fungal counts (CFU g^{-1}) ± SD	
		Culture media	
		DRBC	DG18
Pre-fermentation $3.34 \times 10^3 \pm 2.72 \times 10^3$ [a]	U	$3.8 \times 10^3 \pm 4.0 \times 10^3$ [ab]	$2.5 \times 10^3 \pm 2.9 \times 10^3$
	L	$3.3 \times 10^3 \pm 6.6 \times 10^3$ [a]	$4.2 \times 10^4 \pm 1.0 \times 10^5$
	LT	$4.1 \times 10^3 \pm 5.5 \times 10^3$ [b]	$2.1 \times 10^3 \pm 4.1 \times 10^3$
	C	$5.7 \times 10^3 \pm 8.5 \times 10^3$ [b]	$3.9 \times 10^3 \pm 7.0 \times 10^3$
Post-fermentation $5.74 \times 10^4 \pm 1.82 \times 10^4$ [b]	U	$1.3 \times 10^5 \pm 2.8 \times 10^5$ [a]	$2.4 \times 10^4 \pm 3.9 \times 10^4$
	L	$5.2 \times 10^4 \pm 9.1 \times 10^4$ [a]	$4.3 \times 10^4 \pm 1.0 \times 10^5$
	LT	$3.3 \times 10^4 \pm 1.0 \times 10^5$ [a]	$1.4 \times 10^4 \pm 2.5 \times 10^4$
	C	$1.3 \times 10^3 \pm 8.5 \times 10^3$ [a]	$1.8 \times 10^4 \pm 3.1 \times 10^4$

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 ($P < 0.001$** for pre and post-fermentation samples and $P < 0.05$* for U, L, LT and C). The difference between pre and post-fermentation samples was analyzed separately and the different letters indicate differences within rows of the first column. The difference between the sections of the silo was analyzed within the same group of samples (pre or post-fermentation samples).

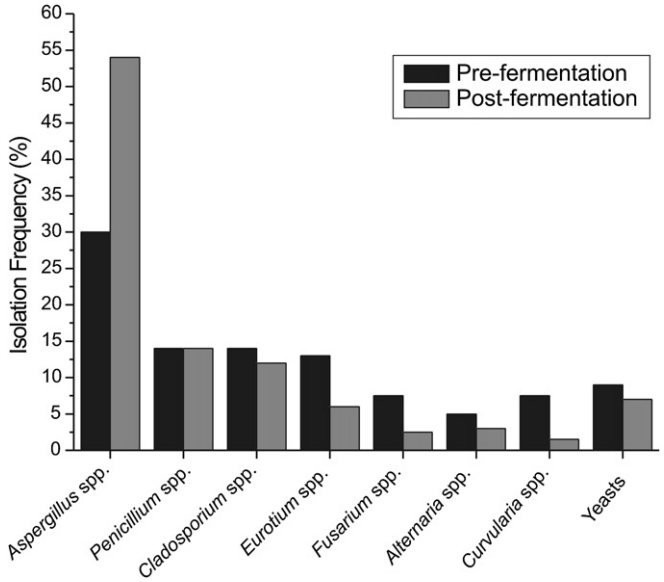

Fig. 1. Isolation frequency (%) of fungal genera isolated from pre and post-fermented corn silage samples.

were higher in post-fermentation samples ($F_{1,5} = 15.55$; $P < 0.05$). The LT section showed levels higher than the rest in for the three mycotoxins ($P < 0.05$). There was no significant difference between OTA levels in pre and post-fermentation samples. Some post-fermentation samples showed AFB_1 levels that exceeded the permitted levels (20 μg g^{-1}) (GMP, 2008). Ochratoxin A, FB_1 and DON levels found in pre and post-fermentation samples were all below recommended limits. However, an important frequency of contamination with these mycotoxins was observed (Table 2).

4. Discussion

Physical evaluation of the samples showed no difference in DM %, pH and a_W values for pre and post-fermentation samples. The values obtained were comparable to the ones obtained by González Pereyra et al. (2008) who found an average DM% value of 47.17% and pH values that varied from 3.88 to 5.89 in post-fermented corn silage samples collected in Argentina. On the contrary, a_W values in our samples were similar than that obtained by González Pereyra et al. (2008).

Total fungal counts present in 25% of pre-fermented and 70% of post-fermented samples exceeded the limit recommended as a quality standard for animal feeds (1×10^4 CFU g^{-1}) (GMP, 2008). Total fungal counts increased during the fermentation process being higher in post than in pre-fermentation samples. *Fusarium* spp. counts on NS medium were low and even under the limit of detection in some samples. Total fungal counts can be compared to the results obtained by González Pereyra et al. (2008) who found that 90% of their samples had counts over 1×10^4 CFU g^{-1} and Reyes-Velázquez et al. (2008) who found counts that varied from 1.7×10^3 to 9.0×10^8 CFU g^{-1}, in corn silage samples from Argentina and Mexico, respectively. Mycobiota isolated from corn silage in our study was comparable to species found by other authors in the same substrate in Argentina, Brazil, France and Egypt being *Aspergillus* and *Penicillium* the most frequently isolated genera (González Pereyra et al., 2008; Moreno et al., 2009; Richard et al., 2007; El-Shanawany et al., 2005). 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 abundance in post-

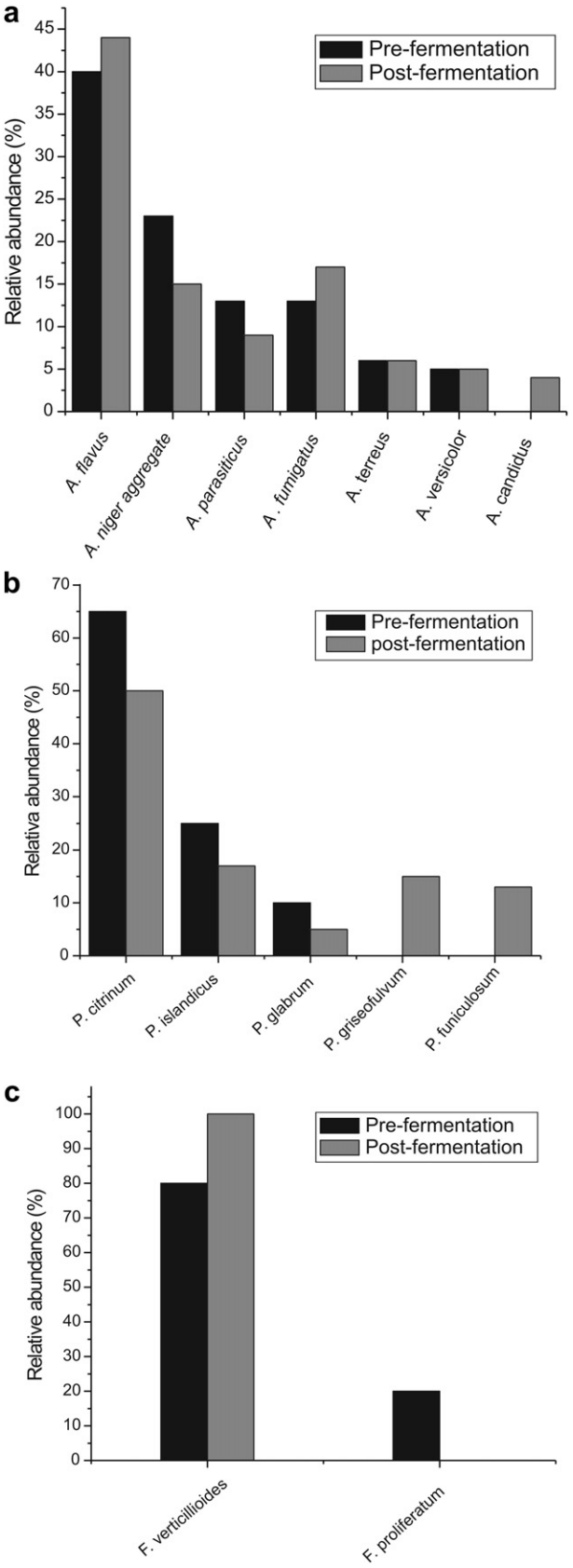

Fig. 2. Relative abundance (%) of a) *Aspergillus*, b) *Penicillium* and c) *Fusarium* species isolated from pre and post-fermented corn silage samples.

Table 2
Mycotoxin levels and contamination frequency of corn silage samples before and after fermentation (pre and post-fermentation).

Samples	Mycotoxin	Silo section	Contamination frequency (%)	Level range (μg g^{-1})	Mean ± SD	Samples exceeding regulation limits (%)
Pre-fermentation	AFB_1^A	U	10.34	0.002–0.012[a]	0.008 ± 0.003	–
		L	17.24	0.004–0.011[a]	0.007 ± 0.003	–
		LT	12.07	0.003–0.011[ab]	0.008 ± 0.003	–
		C	12.07	0.003–0.011[b]	0.007 ± 0.004	–
	OTA^A	U	8.62	0.001–0.028	0.009 ± 0.011	–
		L	10.34	0.007–0.040	0.027 ± 0.010	–
		LT	10.34	0.007–0.040	0.026 ± 0.011	–
		C	8.62	0.001–0.028	0.008 ± 0.011	–
	FB_1^A	U	17.24	0.10–3.03[a]	1.40 ± 1.33	–
		L	15.52	0.20–4.03[a]	2.41 ± 1.18	–
		LT	18.97	0.74–3.97[a]	2.33 ± 0.97	–
		C	15.52	0.31–2.99[a]	1.59 ± 1.09	–
	DON^A	U	17.24	0.10–3.03[a]	1.54 ± 1.33	–
		L	17.24	0.11–4.03[a]	2.10 ± 1.3	–
		LT	31.04	0.74–4.14[ab]	2.54 ± 1.10	–
		C	20.69	0.27–3.03[b]	1.53 ± 1.13	–
Post-fermentation	AFB_1^B	U	17.24	0.012–0.052[a]	0.033 ± 0.013	15.52
		L	13.79	0.006–0.061[ab]	0.030 ± 0.020	8.62
		LT	12.07	0.010–0.056[b]	0.035 ± 0.017	10.34
		C	12.07	0.002–0.054[b]	0.033 ± 0.017	10.34
	OTA^A	U	8.62	0.007–0.027	0.018 ± 0.008	–
		L	8.62	0.020–0.040	0.029 ± 0.007	–
		LT	22.41	0.001–0.040	0.007 ± 0.011	–
		C	10.34	0.003–0.004	0.003 ± 0.000	–
	FB_1^B	U	17.24	0.22–10.00[a]	4.25 ± 2.82	–
		L	15.52	0.27–8.92[ab]	4.04 ± 3.28	–
		LT	15.52	2.01–9.72[b]	5.44 ± 2.55	–
		C	15.52	0.30–3.42[b]	1.60 ± 1.20	–
	DON^B	U	17.24	0.22–10.00[a]	4.25 ± 2.82	–
		L	18.97	0.27–8.92[ab]	3.67 ± 3.08	–
		LT	39.66	0.11–9.72[b]	4.77 ± 2.83	–
		C	24.14	0.15–3.42[b]	1.61 ± 1.12	–

Sampled sections of the silo: U: upper; L: lower; C: center; LT: laterals.
Limits: 0.02 μg g^{-1} AFB_1 (GMP, 2008), 0.25 μg g^{-1} OTA, 50 μg g^{-1} FB_1, 12 μg g^{-1} DON (EU, 2006).
[A,B,a,b]Values indicated with different letters are significantly different according to ANOVA ($P < 0.05^*$ for pre and post-fermentation samples) and LSD test ($P < 0.05^*$ for U, L, C and LT). The difference between pre and post-fermentation samples was analyzed separately for each toxin and indicated with A, B. The difference between the sections of the silo was analyzed for each toxin within the same group of samples (pre or post-fermentation samples) and was indicated with a, b.

fermented silage samples was higher that in pre-fermented samples. In contrast, *P. griseofulvum*, *P. funiculosum* and *F. graminearum* were isolated only in pre-fermented samples. Most *A. flavus* and *A. parasiticus* and strains isolated in this study were able to produce AFB_1 *in vitro*. Ochratoxigenic strains of *A. niger* aggregate (22.37%) were also isolated from the samples increasing the risk of contamination of the substrate. Likewise, El-Shanawany et al. (2005) isolated ochratoxigenic *A. niger* aggregate and aflatoxigenic *A. flavus* and *A. parasiticus* strains among other toxigenic species from corn silage samples collected in Egypt.

Aflatoxin B_1 contamination was detected in all sampled areas of the silos – U, L, LT and C – and, after the silo was cut, each layer showed several samples that exceeded the permitted limit for AFB_1 in cattle feeds (20 μg g^{-1}) (GMP, 2008). Aflatoxin B_1 contamination frequency and levels were higher on post-fermentation silage samples when compared to pre-fermented samples. This suggests that *Aspergillus* section *Flavi* and AFB_1 contamination was enhanced during storage. Ensiling process is supposed to minimize fungal contamination since pH is reduced to an unfavorable acid condition and oxygen is consumed to anaerobiosis. However, bad storing condition and practices during the ensiling process or even after a silo is cut for feedout, can lead to this kind of contamination. González Pereyra et al. (2008) reported that pre-fermentation corn silage samples showed no AFs contamination, whereas post-fermentation 17% of the samples were naturally contaminated with AFB_1 and 67% of them showed levels over 20 μg kg^{-1}.

In the present study, OTA levels did not exceed the limit proposed for cereal derived feedstuffs (0.25 μg g^{-1}) (EU, 2006). These results thus concur with other authors who did not detect significant levels of this mycotoxin in corn silage (Richard et al., 2007, 2009; El-Shanawany et al., 2005). Reyes-Velázquez et al. (2008) found 4.4–5.8 μg g^{-1} OTA in corn silage samples from Mexico.

Fumonisin B_1 and DON were also detected in levels that were all below the recommended limit by the UE for ruminant feedstuffs (50 μg g^{-1} for FB_1 and 12 μg g^{-1} for DON) (EU, 2006). Fumonisin B_1 and DON levels increased during storage, being higher in post-fermentation samples. Likewise, González Pereyra et al. (2008) reported that FB_1 and DON levels were higher in post-fermentation silage when compared to pre-fermentation samples. However, FB_1 levels in these samples were up to 2.49 μg g^{-1}. Similarly, Kim et al. (2004) detected FB_1 levels in corn silage up to 1.82 μg g^{-1}. Even though cattle do not appear to be extremely sensitive to FBs and FB_1 levels found in silage are generally low, most silage is made from annual crops and there can be variations in quality from year to year. Moreover, the presence of *F. verticillioides* in this substrate increases the risk of significant contamination in poorly managed silages. Deoxynivalenol levels found in our study were comparable to those found by other authors (Richard et al., 2007; Reyes-Velázquez et al. 2008).

The present study reveals toxigenic fungi and their mycotoxins are present in corn silage intended for bovine feeding in Brazil. Determination of mycotoxin levels and their frequency of occurrence in corn silage as well as in other raw materials and feedstuffs is important to provide information so that the assessments of risk for animal feed and livestock environment can be made.

Acknowledgments

This study was carried out with grants from CAPES-DS and FAPUR/UFRRJ (Brazil), and PICT-CNPq (Argentina).

References

Association of Official Analytical Chemists (AOAC), 2000. Official Methods of Analysis. AOAC International, seventeenth ed. Maryland, USA, Gaithersburg.

Bennett, J.W., Klich, M., 2003. Mycotoxins. Clinical Microbiology Reviews 16, 497–516.

Black, R.D., McVey, D.S., Oehme, F.W., 1992. Immunotoxicity in the bovine animal: a review. Veterinary and Human Toxicology 34 (5), 438–442.

Bodine, A.B., Mertens, D.R., 1983. Toxicology, metabolism and physiological effects of aflatoxin in the bovine. In: Diener, U.L., Asquith, R.L., Dickens, J.W. (Eds.), Aflatoxin and *Aspergillus flavus* in Corn. Auburn University. Alabama Ag. Exp. Sta., Alabama, USA, pp. 46–50.

Boysen, M.E., Jacobson, K.G., Schnurer, J., 2000. Molecular identification of species from the *Penicillium roqueforti* group associated with spoiled animal feed. Applied and Environmental Microbiology 66, 1523–1526.

Council for Agricultural Science and Technology (CAST), 2003. Mycotoxins: Risks in Plant, Animal and Human Systems, Task Force Report N°139, Ames, Iowa, USA.

Cleveland, T.E., Dowd, P.F., Desjardins, A.E., Bhatnagar, D., Cotty, P.Y., 2003. United States Department of Agriculture-Agricultural Research Service research on pre-harvest prevention of mycotoxins and mycotoxigenic fungi in US crops. Pest Management Science 59 (5–6), 629–642.

Corbett, W.T., Brownie, C.F., Hagler, S.B., Hagler Jr., W.M., 1988. An epidemiological investigation associating aflatoxin M1 with milk production in dairy cattle. Veterinary and Human Toxicology 30 (1), 5–8.

Dos Santos, V.M., Dorner, J.W., Carreira, F., 2003. Isolation and toxigenicity of *Aspergillus fumigatus* from moldy silage. Mycopathologia 156 (2), 133–138.

El-Shanawany, A.A., Eman Mostafa, M., Barakat, A., 2005. Fungal populations and mycotoxins in silage in Assiut and Sohag governorates in Egypt, with a special reference to characteristic Aspergilli toxins. Mycopathologia 159, 281–289.

European Union (EU), 2006. Commission recommendation of 17 august 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding (2006/576/EC). Official Journal of European Union. Available on: URL: <http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:229:0007:0009:EN:PDF> (accessed on 17.08.06.).

Frisvad, J.C., Thrane, U., Samson, R.A., Pitt, J.L., 2006. Important mycotoxins and the fungi which produce them. Advances in Experimental Medicine and Biology 571, 3–31.

Geisen, R., 1996. Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. Systematic and Applied Microbiology 19 (3), 388–392.

GMP, March 2008. Certification Scheme Animal Feed Sector 2006, Including Residue Standards. Version 28.

González Pereyra, M.L., Alonso, V.A., Sager, R., Morlaco, M.B., Magnoli, C.E., Astoreca, A.L., Rosa, C.A.R., Chiacchiera, S.M., Dalcero, A.M., Cavaglieri, L.R., 2008. Fungi and selected mycotoxins from pre- and post- fermented corn silage. Journal of Applied Microbiology 104 (4), 1034–1041.

Howard, P.C., Eppley, R.M., Stack, M.E., Warbritton, A., Voss, K.A., Lorentzen, R.J., Kovach, R.M., Bucci, T.J., 2001. Fumonisin B_1 carcinogenicity in a two-year feeding study using F344 rats and B6C3F1 mice. Environmental Health Perspectives 109 (2), 277–282.

Kalac, P., Woolford, M.K., 1982. A review of some aspects of possible associations between the feeding of silage and animal health. The British Veterinary Journal 138, 305–320.

Kim, E.K., Maragos, C.H., Kendra, D.F., 2004. Liquid chromatography determination of fumonisins B_1, B_2 and B_3 in corn silage. Journal of Agricultural and Food Chemistry 52, 196–200.

Klich, M.A., 2002. Identification of Common Aspergillus Species. CBS, Utrecht, Netherlands.

Marasas, W.F.O., 2001. Discovery and occurrence of the fumonisins: a historical perspective. Environmental Health Perspectives 109 (2), 239–243.

Marasas, W.F.O., Miller, J.D., Riley, R.T., Visconti, A., 2000. Environmental Health Criteria 219. Fumonisin B1. International Programme on Chemical Safety, World Health Organization, Geneva, Switzerland.

Moreno, E.C., García, G.T., Ono, M.A., Vizoni, E., Kawamura, O., Hirooka, E.Y., Ono, E.Y.S., 2009. Co-occurrence of mycotoxins in corn samples from the northern region of Paraná state, Brazil. Food Chemistry 116 (1), 220–226.

Nelson, P.E., Toussoun, T.A., Marasas, W.F.O., 1983. Fusarium Species: An Illustrated Manual for Identification. The Pennsylvania State University Press, University Park and London, UK.

Nout, M.J.R., Bouwmeester, H.M., Haaskma, J., Van Dijk, H., 1993. Fungal growth in silages of sugarbeet press pulp and corn. The Journal of Agricultural Science 121, 323–326.

Ohyama, Y., Masaki, S., Hara, S., 1975. Factors influencing aerobic deterioration of silages and changes in chemical composition after opening silos. Journal of the Science of Food and Agriculture 26, 1137–1147.

Osweiler, G.D., Kehrli, M.E., Stabel, J.R., Thurston, J.R., Ross, P.F., Wilson, T.M., 1993. Effects of fumonisin-contaminated corn screenings on growth and health of feeder calves. Journal of Animal Science 71, 459–466.

Pitt, J.I., Hocking, A.D. (Eds.), 1997. Fungi and Food Spoilage, second ed. Blackie Academic Press, London.

Quinn, G.P., Keough, M.J. (Eds.), 2002. Experimental Design Data Analysis for Biologists. Cambridge University Press, Cambridge, UK.

Reyes-Velázquez, W.P., Espinoza, V.H.I., Rojo, F., Jiménez-Plasencia, C., Palacios, E.L., Hernández-Góbora, J., Ramírez-Álvarez, A., 2008. Occurrence of fungi and mycotoxins in corn silage, Jalisco state, Mexico. Revista Iberoamericana de Micología 25, 182–185.

Rice, L.G., Ross, P.F., 1994. Methods for detection and quantitation of fumonisins in corn, cereal products and animal excreta. Journal of Food Protection 57, 536–540.

Richard, E., Heutte, N., Bouchart, V., Garon, D., 2009. Evaluation of fungal contamination and mycotoxin production in maize silage. Animal Feed Science and Technology 148, 309–320.

Richard, E., Heutte, N., Sage, L., Pottier, D., Bouchart, V., Lebailly, P., Garon, D., 2007. Toxigenic fungi and mycotoxins in mature corn silage. Food Chemical Toxicology 45, 2420–2425.

Rodrigues, P.B., Freitas, R.T.F., Fialho, E.T., Silva, H.O., Tascisio Morais Gonçalves, T.M., 2002. Digestibilidade dos nutrientes e desempenho de suínos em crescimento e terminação alimentados com rações à base de milho e sorgo suplementadas com enzimas. Revista Brasileira de Milho e Sorgo 1 (2), 91–100.

Samson, R.A., Frisvad, J.C., 2004. Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and air-borne terverticillat Penicillia and their mycotoxins. Studies in Mycology 49, 1–174.

Samson, R.A., Hockstra, E.S., Frisvad, J.C., Filtenborg, O., 2000. Introduction to Food and Airborne Fungi. Centaalbureau Voorschimmelculturs-Utrecht Ponson Looyen. Wageningen Press, The Netherlands.

Scudamore, K.A., Nawaz, S., Hetmanski, M.T., 1998. Mycotoxins in ingredients of animal feeding stuffs: II. Determination of mycotoxins in maize and maize products. Food Additives and Contaminants 15, 30–55.

Smith, G.W., Constable, P.D., Foreman, J.H., Eppley, R.M., Waggoner, A.L., Tumbleson, M.E., Haschek, W.M., 2002. Cardiovascular changes associated with intravenous administration of fumonisin B1 in horses. American Journal of Veterinary Research 63, 538–545.

Soares, L.M.V., Rodeigues-Amaya, D., 1989. Survey of aflatoxins, ochratoxin A, zearalenone, and sterigmatocystin in some Brazilian foods by using multi-toxin thin-layer chromatographic method. Journal – Association of Official Analytical Chemists 72 (1), 22–26.

Téren, J., Varga, J., Hamari, Z., Rinyu, E., Kevei, F., 1996. Immunochemical detection of ochratoxin A in black *Aspergillus* strains. Mycopathologia 134 (3), 171–176.