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Original

Mycobiota and mycotoxins in fermented feed, wheat grains and corn grains in Southeastern Buenos Aires Province, Argentina

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ABSTRACT

Wheat (as bran) and corn (as dry grain or fermented feed) are main ingredients of feedstuffs used in local cattle and pig farms in the South of the Buenos Aires Province (Argentina). Therefore, determining mycobiota and mycotoxins in wheat and corn is of prime importance for developing feed management techniques to optimise animal production and to minimize toxicity. Then, a mycological survey was carried out in the Southeastern part of the Buenos Aires Province, in order to identify the mycobiota and the main mycotoxins present in fermented feed, wheat grain and corn grain samples. Samples were cultured for fungal quantification, isolation and identification, and analysed for deoxynivalenol (DON), zearalenone (ZEA), T-2 toxin and aflatoxins (AFLA). *Penicillium* (74%), *Aspergillus* (32%) and *Scopulariopsis* (21%) were the prevalent genera in fermented feed. *Penicillium* (70%), *Fusarium* (47%) and *Aspergillus* (34%) were the most frequent fungi isolated from corn. *Penicillium* (42%), *Fusarium* (27%) and *Alternaria* (25%) were the most frequently recovered genera from wheat. DON was detected in 59% of the corn samples, in 45% of the wheat samples and in 38% of the silage samples. ZEA was detected in 36% of the corn samples, in 49% of the wheat samples and in 16% of the silage samples. T-2 toxin and aflatoxin B1 were each detected in 4% of the corn samples. Eighteen percent of the fermented feed samples showed T-2 contamination. Fermented feed and wheat samples were negative for AFLA.
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Micobiota y micotoxinas en piensos, granos de trigo y granos de maíz en el sudeste de la provincia de Buenos Aires, Argentina

Palabras clave:
Aflatoxinas
Alimentos fermentados
Aspergillus
Cereales
Desoxinivalenol
Fusarium
Penicillium
Zearalenona
Toxina T-2

RESUMEN

El trigo (como afrechillo) y el maíz (como grano seco o alimento fermentado) son ingredientes empleados como alimento para el ganado bovino y porcino en la zona sur de la provincia de Buenos Aires (Argentina). Determinar la micobiota y las micotoxinas presentes en estos alimentos es de suma importancia para establecer técnicas de control de los mismos, optimizar la producción animal y minimizar su toxicidad. Por ende, en el sudeste de la provincia de Buenos Aires se llevó a cabo un estudio para identificar la micobiota y las principales micotoxinas presentes en dichos tipos de alimento. Las muestras fueron sembradas para realizar el recuento, el aislamiento y la identificación de los principales géneros de hongos presentes, y analizadas para detectar las micotoxinas de mayor importancia toxicológica: desoxinivalenol (DON), zearalenona (ZEA), toxina T-2 y aflatoxinas (AFLA). *Penicillium* (74%), *Aspergillus* (32%) y *Scopulariopsis* (21%) fueron los géneros más frecuentes en alimentos fermentados. En muestras de maíz, los géneros más relevantes fueron *Penicillium* (70%), *Fusarium* (47%) y *Aspergillus* (34%), mientras que *Penicillium* (42%), *Fusarium* (27%) y *Alternaria* (25%) fueron los más aislados en el caso del afrechillo de trigo. DON fue detectado en el 59% de las muestras de maíz, en el 45% de las muestras de trigo y en el 38% de las muestras de alimento fermentado. ZEA fue detectada en un 36% de las muestras de maíz, en un 49% de las muestras de trigo y en un 16% de las muestras de alimento fermentado. Las toxinas T-2 y aflatoxina B1 fueron detectadas en un 4% de las muestras de maíz. El 18% de las muestras de alimentos fermentados mostraron contaminación con T-2. Las muestras de alimentos fermentados y de trigo fueron negativas para AFLA.
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Mycotoxins are toxic secondary metabolites produced by filamentous fungi (moulds). Moulds may occur in several types of food destined to animal consumption, including wheat and corn grains or their by-products, as well as in fermented feed (corn silage and high moisture corn). Mycotoxin production may occur in the field, during post harvest, storage, processing or feeding under appropriate environmental conditions. These metabolites are generally associated with fungi belonging to the genera *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium*.[28,32] Toxigenic *Alternaria* and *Fusarium* species are often classified as field fungi, while *Aspergillus* and *Penicillium* species are considered storage fungi.[20] Although more than 100 mycotoxins have been identified, less than 10 are of concern due to their natural occurrence and toxicity.[43]

The most common *Fusarium* mycotoxins are trichothecenes, zearalenone (ZEA) and fumonisins (FUM).[7] The mycotoxins of interest produced by *Aspergillus* spp. include aflatoxins (AFLA) and ochratoxin A (OTA), while *Penicillium* spp. produces OTA, citrinin and patulin among the more important mycotoxins.[20]

The detection of fungi does not necessarily entail the presence of mycotoxins, since mycotoxin production depends on various factors such as the presence of toxigenic fungi, chemical composition of the substrate, moisture content, relative humidity, temperature and time course of fungal growth. However, high incidence rates of mycotoxin contamination of cereal seeds and animals' feed have been reported worldwide.[33]

The negative effect of mycotoxins on the growth and health of livestock makes them a major problem for many production systems. Symptoms of mycotoxicosis depend on the type of mycotoxin, the amount and duration of the exposure, the age, health and sex of the exposed individual, as well as on dietary status and interactions with other toxins.[1] Low levels of mycotoxins may cause reduction of feed intake and decreased performance, such as lowered milk production or decrease in body weight gain. Moderate levels of contamination of feed frequently result in impaired resistance to infections, increased susceptibility to stress and reduced fertility. High levels of contamination may produce clinical disease, liver and kidney damage, edema, increased blood clotting time and haemorrhaging, as well as altered digestion, absorption and metabolism of nutrients.[24]

Wheat (*Triticum aestivum* L.) and corn (*Zea mays* L.) are the most important cereal crops in Argentina. More than 70% of both commodities are exported, whereas the remaining meets the domestic demand.[18,36]

Data on fungal and mycotoxin contamination in both commodities are available from major production areas of Argentina. *Fusarium graminearum* and deoxynivalenol (DON) have been detected in wheat in several occasions.[6,12,13,34] Surveys carried out in samples from Santa Fe Province, one of the most important agricultural regions of the country, reported the presence of species of the genera *Aspergillus*, *Fusarium*, *Penicillium*, *Scopulariopsis*, and *Trichoderma*, as well as contamination with DON ranging from 0.5 to 2 ppm.[10]

The largest corn producing provinces are (East) Córdoba, (South) Santa Fe and (Northeast) Buenos Aires, which comprise the "Corn Belt", accounting for 80% of the total of maize production of the country. Variable levels of AFLA and ZEA were detected in corn samples from Santa Fe and Buenos Aires Provinces from 1983 to 1994.[35]

A survey carried out in corn from selected sampling areas in Entre Rios Province showed contamination with AFLA, DON, ZEA and FUM in corn harvested in 2003, while only AFLA and FUM were found in samples obtained from 2004.[4] In another study, *Alternaria alternata* was the major species isolated from cereal seeds (including freshly harvested wheat). *Fusarium verticillioides* was the major species isolated from corn.[3]

The Southeastern part of Buenos Aires Province is an important dairy and beef cattle production area. In addition, the number of pig farms has increased in this region over the last years. Wheat (as bran) and corn (as dry grain or fermented feed) are main ingredients of feedstuffs used in local cattle and pig farms. Some farmers plant corn for on-farm livestock demands. After harvest, on-farm silage demands are usually met first, while the remaining corn is harvested for grain in order to be sold for domestic consumption or export.

Lori et al.[21] analysed durum wheat samples from Southern Buenos Aires Province during two consecutive harvests. None was free of *F. graminearum* infection. Moreover, in both years, samples from locations situated in the humid areas showed DON ranging from 0.1 to 8 ppm.

Because the practical absence of data about the fungi involved in the deterioration and the production of mycotoxins in the most important feeds for cattle, the aim of this survey was to quantify and to determine the most common fungal genera and the main mycotoxins present in fermented feed, wheat and corn grains from the Southeastern Buenos Aires Province.

Material and methods

Sample sources

During 2005 and 2006, a total of 56 samples of wheat and 53 of corn seeds collected either from fields or from trucks at the store plants, and 65 samples of fermented feed (corn silage and high moisture corn) submitted to the Toxicology Laboratory at the UNCPBA, were cultured for fungal quantification and isolation, and were analysed for the most important mycotoxins (DON, T-2, ZEA and AFLA). After collection, samples were stored at 4 °C until isolation and identification of the fungi.

Mycological study

Samples (500 g) were ground in a laboratory mill. Portions of 10 g of each sample were homogenized with 90 mL sterile distilled water for 30 min on horizontal shaker. Further dilutions were prepared by transferring 1 mL aliquots of the initial dilution into succeeding dilution bottles containing 9 mL of sterile distilled water. One millilitre portions of these dilutions were placed onto Difco® acid potato dextrose agar (APDA) (Becton Dickinson Co., USA). Plates were incubated at 22 °C in darkness for 5 days, and then under 12 h light and dark cycles for 2 days. Cultures were identified at generic level based on fisiological and morphological characters according to Nelson et al.[26] and Pitt and Hocking.[31] The number of colony-forming units per gram (cfu/g) of feed was determined for each genus. The isolation frequency (Fq) of genera was calculated according to Marassas et al.[22]

Mycotoxin analyses

Twenty five grams of each sample were finely ground and the mycotoxins were extracted with acetonitrile:water (84:16) for 30 min by shaking in a horizontal shaker. Extracts were filtrated through Whatman No. 4 filter paper.

Pure mycotoxin standards were purchased from Sigma Chemical Company (USA). For DON and T-2 toxin, 10 mL of the filtrate was passed through a cleanup column packed with activated charcoal:alumina:celite (7:5:3) and eluted with a 5 mL of

acetonitrile:water (84:16) mixture. After evaporation to dryness in a water bath at 60 °C, the residue was dissolved in 100 μL chloroform. A 10 μL aliquot was applied on silica gel 60 TLC plates (Merk KGaA, Germany) and developed in toluene:ethyl acetate:-formic acid (5:4:1). For DON visualization, plates were sprayed with 20% aqueous solution of aluminium chloride and heated at 115 °C for 10 min. DON became visible as a bright grey fluorescent spot. For T-2 toxin visualization, plates were sprayed with 20% sulphuric acid in water and heated at 115 °C for 10 min. T-2 toxin became visible as a brown spot.[37] Detection limits for DON and T-2 toxin were 0.1 and 0.28 ppm, respectively. For AFLA and ZEA determination, 5 mL on the initial extract was diluted with 5 mL of distilled water and defatted twice with 10 mL of n-hexane and then double extracted with 10 mL of chloroform. The combined chloroform phase was evaporated to dryness in a water bath. The residue was dissolved with 100 μL of chloroform. A 10 μL aliquot was applied on silica gel TLC plates and developed in toluene:ethyl acetate:formic acid (5:4:1). Zearalenone became visible after spraying the TLC plate with 0.7% Fast Violet B in water followed by a pH 9 buffer solution (50 mL 0.025 M Na borate and 4.6 mL 0.1 M HCl). Zearalenone was visualized as a purple spot.[37] The detection limits for AFLA and ZEA were 2.5 and 80 ppb, respectively. Corn samples positive to ZEA and DON by TLC were quantified by HPLC.

Zearalenone was quantified by reversed-phase HPLC with fluorescent detection. One millilitre of the original extract was cleaned up by immunoaffinity column (Romer Labs Diagnostic GmbH, Austria) following methodology supplied by the manufacturer. The chromatographic conditions consisted of a mobile phase of a mixture of acetonitrile:water:methanol (32:40:28) eluted at a flow rate of 1 mL/min. The column was a 4.60 mm × 250 mm reversed-phase LUNA 5 μm C_{18} (Phenomenex, USA). Fluorescent detection (spectrofluorometric detector RF-10; Shimadzu, Japan) was at an excitation wavelength of 236 nm and an emission wavelength of 418 nm.

DON was quantified by reversed-phase HPLC with UV detection. The chromatographic conditions consisted of a mobile phase of a mixture of acetonitrile:water (10:90) eluted at a flow rate of 0.5 mL/min. The column was a 3.0 mm × 250 mm reversed-phase Synergy Hydro-RP 4 μm C_{18} (Phenomenex). The HPLC system consisted of a Gilson Pump model 307 and a Gilson UV detector model 129 set at 222 nm wavelength.[41]

Results

Eleven genera of filamentous fungi were isolated from the fermented feed samples (Table 1). *Penicillium* (74%), *Aspergillus* (32%) and *Scopulariopsis* (21%) were the most common genera. *Fusarium* was isolated in 15% of such samples. High concentrations (10^6 ufc/g) of *Penicillium* and *Aspergillus* were found in 77% and 62% of fermented feed samples, respectively. Seven genera were isolated from corn samples. *Penicillium* (70%), *Fusarium* (47%) and *Aspergillus* (34%) were the most frequent and abundant genera (Table 1). Ten genera of moulds were isolated from wheat samples (Table 1). *Penicillium* was the most frequent (42%). Twenty nine percent of samples positive for *Penicillium* showed around 10^6 cfu/g of feed. *Fusarium* (27%) and *Alternaria* (25%) were the second and the third more frequent in wheat samples.

DON was the most common mycotoxin in all samples (Table 2). DON levels in corn grains ranged from 0.24 to 1 ppm. ZEA was the second most common mycotoxin (Table 2). ZEA levels in corn grains ranged from >0.1 to 1.56 ppm. T-2 toxin was detected in 18% of the fermented feed samples, in 4% of the corn samples and in 2% of wheat samples (Table 2).

Aflatoxin B1 was detected in 2 out of 58 corn samples. Fermented feed and wheat samples were negative for AFLA.

Discussion

Penicillium, Aspergillus and *Scopulariopsis* were the most prevalent genera in fermented feeds produced in the Southeast of the Buenos Aires Province. A survey carried out in the central

Table 2
Mycotoxins in the studied samples

Mycotoxins	Fermented feed ($n = 38$)		Corn grain ($n = 58$)		Wheat grain ($n = 45$)	
	Samples	Fq	Samples	Fq	Samples	Fq
DON	13	34	34	59	22	49
Zearalenone	6	16	21	36	22	49
T-2	7	18	2	4	1	2
Aflatoxins	0	0	2	4	0	0

Table 1
Frequency and concentration of different genera of fungi present in samples of fermented feed, corn and wheat seeds from the Southeastern Buenos Aires Province during 2005 and 2006

Genera of fungi	Fermented feed ($n = 65$)							Corn ($n = 53$)						Wheat ($n = 56$)					
			cfu/g of feed							cfu/g of feed						cfu/g of feed			
	n	Fq[a]	10^2	10^3	10^4	10^5	10^6	n	Fq[a]	10^3	10^4	10^5	10^6	n	Fq[a]	10^3	10^4	10^5	10^6
Penicillium	48	74	1	4	1	5	37	37	70	–	7	9	21	24	42	–	6	11	7
Aspergillus	21	32	1	1	3	3	13	18	34	–	4	7	7	7	12	–	1	5	1
Scopulariopsis	14	21	3	–	1	1	9	–	–	–	–	–	–	9	16	–	8	1	–
Fusarium	10	15	1	1	2	2	4	25	47	–	5	13	7	15	27	–	8	4	3
Cladosporium	8	12	–	–	4	2	2	5	9	–	1	2	2	7	12	–	2	2	3
Alternaria	5	8	–	1	1	–	3	2	4	–	–	1	1	14	25	–	4	4	6
Geotrichum	4	6	–	–	1	1	2	–	–	–	–	–	–	–	–	–	–	–	-
Mucor	2	3	–	–	–	1	1	3	6	–	3	–	–	2	3	–	1	–	1
Acremonium	1	1	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	-
Moniliella	2	3	–	–	–	1	1	–	–	–	–	–	–	–	–	–	–	–	-
Trichoderma	1	1	–	–	–	–	1	1	–	–	–	1	–	–	–	–	–	–	-
Epicoccum	–	–	–	–	–	–	–	1	2	–	–	1	–	3	3	–	1	2	–
Monascus	–	–	–	–	–	–	–	–	–	–	–	–	–	1	1	–	1	–	-
Rhizopus	–	–	–	–	–	–	–	–	–	–	–	–	–	1	1	–	–	-	1

[a] Frequency, measured as percentage.

area of Santa Fe Province (31°18′S, 61°55′W), showed that *Aspergillus* and *Byssochlamys* were the most common moulds in corn silage, while *Penicillium* was less frequent.[10] In other study, *Aspergillus* (78%) and *Fusarium* (62%) were the most frequent genera in corn silage from Córdoba Province (33°18′S, 64°38′W).[15] Differences in geographical and environmental conditions might be responsible for differences in fungal distribution and concentration observed among different locations. However, our results agree with those previous reports indicating that *Penicillium* and *Aspergillus* were the most prevalent moulds isolated from fermented feed in USA[38,39] and in Europe.[9,17,29]

Ensiling is one of the oldest processes applied in feed preservation to prevent the growth of microorganisms, including fungi.[29] However, according to Seglar[38] silages are heavily infested with moulds when total fungal populations exceed 10^5 cfu/g of feed silage. These levels of contamination may affect the feeds by decreasing their nutrient value, the palatability, or due to the adverse effect of mycotoxins on the animal health. The high frequency and abundance of *Penicillium* and *Aspergillus* at the present study could be due to incorrect silage making and conservation. *Fusarium* was isolated in 15% of silage samples. According to Pelhate,[29] *Fusarium* is present at harvesting as a result of field infection, or by contamination at the time of silage making, and cannot longer survive once oxygen becomes depleted during fermentation. This could account for the low frequency of *Fusarium* observed in the silage samples analysed in this study. In contrast, González Pereyra et al.[15] found *Fusarium* as a prevalent mould in the post-fermented feed (>60%). *Penicillium*, *Fusarium* and *Aspergillus* were the most frequent genera in corn samples. In a previous study, *Fusarium* and *Penicillium* were the most prevalent taxa of the internal seed-borne mycobiota in corn seed samples obtained from five different production regions in Argentina, while *Aspergillus* was found at low frequency.[11,14] *Penicillium*, *Fusarium* and *Aspergillus* produced the greatest contamination in corn, varying from 10^4 to 10^6 cfu/g of feed. These levels exceed the tolerance limit set by the International Commission on Microbiological Specifications for Foods.[8]

Penicillium was the most prevalent and abundant genus in wheat samples. A similar incidence of *Penicillium* (43%) was observed in Australia.[2] In other study, members of the genus *Alternaria* and *Fusarium* were highly prevalent, while *Penicillium* was present in a reduced number of samples in durum wheat collected in 1996 from the Southeastern Buenos Aires Province.[13] The second more frequently isolated fungus from wheat grains was *Fusarium.* Similarly, *Fusarium* was the most frequently recovered genus from wheat harvested from different regions of Argentina.[12] A high incidence of *Alternaria* was observed in wheat grain samples. Members of the genus *Alternaria* were the major fungi isolated from freshly harvested wheat from different geographical areas of the country.[3] Evidence is also available elsewhere on cereal grains contamination by the genera *Penicillium* and *Alternaria*.[23] *Alternaria* mycotoxins such as tenuazonic acid, alternariol and alternariol-methyl-ether have been detected in wheat grain in Australia[42] and China.[19] Chemical analyses of the wheat samples will be needed in the future to determine whether *Alternaria* mycotoxins naturally occur in wheat harvested in the region submitted at study herein. The presence of *Aspergillus* in the wheat samples at the present study was lower than values reported in surveys from other parts of the world, where *Aspergillus* ssp. and *Penicillium* spp. are the toxigenic fungi most frequently isolated from wheat.[2] DON was the most common mycotoxin in all samples. Similar results have been reported by other authors. Eighty percent of wheat samples from Córdoba Province and 45% of durum wheat samples from the Southeastern part of the Buenos Aires Province showed DON contamination.[6,13] In another study, DON was found in 55% and 78.2% of durum wheat samples harvested in two consecutive years, from different locations in the Buenos Aires Province.[21] These results also agree with international data, indicating that DON is the most common mycotoxin detected in seeds and grain by-products.[30] DON levels in corn samples ranged from >0.24 to 1.8 ppm. Argentina has not yet issue regulatory guidance for *Fusarium* mycotoxins in feeds. The US Food and Drug Administration (USFDA) have established advisory levels for DON in seeds and grain by-products intended for animal feed. For pigs, a grain or a grain by-product containing 5 ppm of DON should not exceed 20% of the diet, and for cattle it is 10 ppm.[40] Locally, corn comprises the 80% of the pig diet; therefore levels of DON found at the present study (1.8 ppm) could represent a threat for pig nutrition.

Numerous feeding studies and surveys with dairy cattle have not been able to conclusively show a negative cause–effect relationship between DON ingestion and production problems. In Argentina, pasture-based feeding systems for dairy cattle are usually supplemented with concentrates or conserved forages. Corn represents only 3–4% of the diet. Thus, levels of DON detected in corn samples seem not to represent a risk for dairy cattle in this production region. High incidences of DON in silage have been reported from Europe and the US (summarized by Gotlieb[16] and Oldenburg[27]). In the present study, 34% of the fermented feed samples analysed contained detectable levels of DON, while only 15% of samples were positive to *Fusarium*. Fungal infestation and mycotoxin formation before harvest followed by inhibition of *Fusarium* in silage may explain these results.

Zearalenone was the second more common mycotoxin. Similar to DON, ZEA is a frequent contaminant of ensiled feed.[43] In Argentina, the presence of ZEA and DON in silage has been recently reported in corn silage from the central part of this country.[15] The levels of ZEA found in corn (>0.10–1.56 ppm) are known to produce reproduction problems in pigs (reviewed by Morgavi and Riley[25] and Zinedine et al.[44]) and cattle.[5] Signs of hyperestrogenism and reduced fertility in pigs due to ZEA have been frequently reported in this region.

T-2 toxin was detected in 18% of fermented feed samples and in 4% of corn samples. To our knowledge, this constitutes the first report of T-2 contamination in corn (as silage and seeds) in Argentina. T-2 toxin was also found in 2% of wheat samples. In a previous study, T-2 toxin was found in 20 out of 261 wheat samples.[34]

Aflatoxin B1 was detected in only 2 out of 58 wheat samples, while it was not found in fermented feed or corn samples. In Argentina, contamination with aflatoxins is more common in cereals from the Central and Northern provinces where the climate favours *Aspergillus flavus* and *A. parasiticus* growth.[31] Although the present survey covered a small area, there is an unequivocal evidence that DON, ZEA and T-2 toxin occur in corn and wheat seeds and fermented feed produced in the Southeastern region of the Buenos Aires Province, and under certain conditions this fact may represent a risk for animal health and production.

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