



## Food Microbiology

# Fusarium and Aspergillus mycotoxins contaminating wheat silage for dairy cattle feeding in Uruguay



Agustina del Palacio, Lina Bettucci, Dinorah Pan\*

Universidad de la República, Facultad de Ciencias-Facultad de Ingeniería, Montevideo, Uruguay

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## ABSTRACT

Wheat is one of the most important cultivated cereals in Uruguay for human consumption; however, when harvest yields are low, wheat is usually used in ensiling for animal feeding. Ensiling is a forage preservation method that allows for storage during extended periods of time while maintaining nutritional values comparable to fresh pastures. Silage is vulnerable to contamination by spoilage molds and mycotoxins because ensilage materials are excellent substrates for fungal growth. The aim of the study was to identify the mycobiota composition and occurrence of aflatoxins and DON from wheat silage. A total of 220 samples of wheat were collected from four farms in the southwest region of Uruguay where silage practices are developed. The main fungi isolated were *Fusarium* (43%) and *Aspergillus* (36%), with *Fusarium graminearum sensu lato* and *Aspergillus* section *Flavi* being the most prevalent species. Aflatoxin concentrations in silo bags ranged from 6.1 to 23.3  $\mu\text{g}/\text{kg}$ , whereas DON levels ranged between 3000  $\mu\text{g}/\text{kg}$  and 12,400  $\mu\text{g}/\text{kg}$ . When evaluating aflatoxigenic capacity, 27.5% of *Aspergillus* section *Flavi* strains produced AFB1, 5% AFB2, 10% AFG1 and 17.5% AFG2. All isolates of *F. graminearum sensu lato* produced DON and 15-AcDON. The results from this study contribute to the knowledge of mycobiota and mycotoxins present in wheat silage.

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## Introduction

Dairy products are one of the main commodities exported by Uruguay. Although pastures constitute the major source for dairy cattle feeding, silage grains are also used, supplying 125 g per liter of milk produced.<sup>1</sup> Ensiling is a forage preservation method that allows for grain storage during extended

periods of time while maintaining a nutritional value comparable with that of fresh pastures. Grain silage storage is based on the chemical processes that occur in vegetable tissues when they are under anaerobic conditions and in the presence of lactic acid bacteria, which leads to a decrease in pH value (4–4.5), thus inhibiting several spoilage microorganisms. However, silage is vulnerable to contamination by spoilage molds because ensiled materials are excellent substrates for

\* Corresponding author.

E-mail: [dpan@fing.edu.uy](mailto:dpan@fing.edu.uy) (D. Pan).

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fungal growth, which are present as spores in the field, especially in soil.<sup>2</sup> Moreover, factors such as excessive moisture or dryness, condensation, heat, poor compression of the materials, leakage of rainwater and insect infestation can lead to undesirable mold contamination, mycotoxin production and reduction of nutritional value.<sup>3–5</sup>

Mycotoxins are low molecular weight products of fungal secondary metabolism, produced mainly by species of *Aspergillus*, *Penicillium* and *Fusarium*. They can cause human and animal disorders when consumed or inhaled.<sup>6,7</sup> Exposure to mycotoxins through contaminated feed is one of the major risk factors to ruminant health.<sup>7,8</sup>

*Aspergillus flavus* and *A. parasiticus* are species that produce aflatoxins (AFs), the most important carcinogenic mycotoxins. Acute aflatoxicosis in cattle has been thoroughly described. Consumption of feed containing AFs may reduce growth rate, decrease milk production, cause liver damage and depress immune function.<sup>9</sup> Among AFs, aflatoxin B1 (AFB1) is the most carcinogenic, and it is classified by the International Agency for Research on Cancer (IARC) as a group 1 human carcinogen.<sup>10</sup> When animals eat foodstuffs containing AFB1, this toxin is metabolized and accumulated in milk as aflatoxin M1 (AFM1).<sup>11</sup> Many studies have demonstrated that AFM1 also displays toxic and carcinogenic effects; therefore it too has been included in IARC group 1.<sup>12</sup>

*Fusarium* spp. are the most frequent wheat contaminant fungi in Uruguay and are particularly prevalent when spring rains occur.<sup>13</sup> Trichothecenes produced by *Fusarium* species alter immune-mediated activities in bovines.<sup>14</sup> This toxin is a potent inhibitor of eukaryotic protein biosynthesis, inducing vomiting, diarrhea, anemia and food refusal in animals.<sup>15–17</sup>

The aims of this study were to evaluate the following: (1) the mycobiota composition of wheat silages collected during a 4 month period; (2) the toxigenic ability of isolates of *Aspergillus* section *Flavi* and *Fusarium graminearum sensu lato* (*F. graminearum* s.l.) and (3) the mycotoxins produced in wheat grain silage.

## Materials and methods

### Samples

Wheat was harvested from cultivated lands of four farms located in the southwest region of Uruguay, mechanically chopped and enclosed within polystyrene bags. Compaction was conducted in November of 2009, and the silo bag was first opened in January 2010. A total of 220 samples of wheat (4 from freshly harvested grains and 216 from stored grain) were analyzed from November of 2009 at harvest time and at 60, 90 and 120 days of ensiling. The removal of material was performed using a cutting machine. Sampling was performed manually through the silos in transects at three levels. From the cut edge, three points from each section of the silo (upper, middle and lower) were sampled at 50 cm horizontal depth. At each time, 1-kg samples from each section were collected. Samples belonging to each silo were homogenized and quartered to obtain 1-kg sub-samples for analysis.

### Physical properties of samples

Twenty-five grams of each sample were dried in an SC1 oven (Siac S.C., Montevideo, Uruguay) to constant weight in a Petri dish at 105 °C, and the moisture content of samples was expressed as percentage values (MC%). Water activity was determined by automated analysis using an Aqualab CX-2 (Decagon Devices, Inc.). pH measurements were performed by adding 90 mL of distilled water to 10 g of silage materials and homogenizing the mixture for 15 min. The homogenized mixture was filtered, and the pH of the filtrate was measured with an Orion Star A211 glass electrode pH meter (Thermo Scientific).

### Analysis of mycobiota

One-hundred wheat grain particles from each sample were placed in Petri dishes containing potato dextrose agar (PDA) and incubated at 25 °C under a 12 h white/12 h black fluorescent light photoperiod.<sup>18,19</sup> The emerging colonies from each particle of wheat were transferred to allow identification by means of macro- and micromorphological characteristics, following conventional mycological methods.<sup>20–26</sup>

To confirm morphological identifications of the isolates of *F. graminearum* s.l. specific PCR was performed using the primers Fg16F and Fg16R.<sup>27</sup> The ITS region and parts of the  $\beta$ -tubulin and calmodulin genes were amplified to confirm the identification of *Aspergillus* section *Flavi* isolates.<sup>28</sup>

### Determination of mycotoxins in silages samples

The quantitative analyses of DON and aflatoxins in samples were performed using competitive enzyme-linked immunosorbent assays (ELISA) using the Ridascreen® Fast DON and Ridascreen® Fast Aflatoxin SC (R-Biofarm AG) commercial kits. The protocols for extraction and quantification were performed according to manufacturer recommendations. The detection limits of the technique were 200  $\mu$ g/kg for DON and 2  $\mu$ g/kg for aflatoxins.

### Toxigenic capacity of fungal isolates

The ability of potentially toxigenic isolates to produce mycotoxins *in vitro* was tested. Aflatoxin analyses were performed using the methodology described by Bragulat et al.<sup>29</sup> *Aspergillus* section *Flavi* strains isolated from silage samples were incubated in Petri dishes of Coconut Extract Agar (CEA) (20% desiccated coconut; 1.5% agar) and incubated at 25 °C for 14 days in the dark. Then, 3 plugs were cut from each Petri dish and 1 mL of methanol was added. After 1 h in methanol, an aliquot (200  $\mu$ L) was derivatized with 700  $\mu$ L trifluoroacetic acid:acetic acid:water (20:10:70, v/v). The derivatized solution was analyzed using a reversed phase HPLC, consisting of a Shimadzu LC-10ADvp pump, a RF-10AxI fluorescence detector (Shimadzu; excitation and emission wave length of 360 nm and 440 nm, respectively), and a C<sub>18</sub> reversed-phase column (150 mm  $\times$  4.6 mm i.d., 5  $\mu$ m particle size; Nucleodur®, Macherey-Nagel, Düren, Germany) connected to a precolumn Security Guard (8 mm  $\times$  4 mm i.d., 5  $\mu$ m particle size; Nucleodur®, Macherey-Nagel, Düren,

Germany). The mobile phase was water:methanol:acetonitrile (4:1:1, v/v/v) at a flow rate of 1.5 mL min<sup>-1</sup>. The injection volume was 20 µL. Aflatoxin production was measured in ng g<sup>-1</sup> of culture medium. The limit of detection was 1 ng g<sup>-1</sup> of B1 and G1 and 0.8 ng g<sup>-1</sup> of B2 and G2 (Trilogy Analytical Laboratory Inc., Washington, MO).

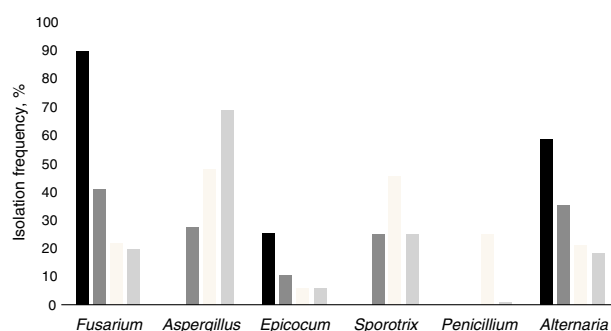
The capacity of *F. graminearum* s.l. isolates to produce trichothecene was determined using multiplex PCR experiments<sup>30</sup> and as follows: 25 g of long grain rice was placed in a 250 mL Erlenmeyer flask and 10 mL of distilled water was added. The flasks were autoclaved twice during 2 consecutive days at 121 °C for 20 min. Each flask was inoculated with a 7-day-culture-carnation leaf agar (CLA) plug and incubated in the dark for 28 days at 25 °C. At the end of the incubation period, the contents of the flask were dried at 50 °C for 24 h and then milled. Fifteen grams of the dried sample were then extracted with 40 mL acetonitrile:methanol (14:1, v/v), shaken for 2 h at 150 rpm and then filtered through Whatman N° 1 filter paper. A syringe (3 mL capacity) plugged with glass wool and dry-packed with alumina:carbon (20:1, w/w, 500 mg) was used as a mini-cleanup column. A 2 mL aliquot of extract was applied to the column, allowed to drain under gravity, and the eluent collected. The column was washed with 500 µL acetonitrile:methanol:water (80:5:15, v/v), and the combined eluents were evaporated to dryness under N<sub>2</sub> at 50 °C. The cleaned residue was dissolved in 500 µL toluene:acetonitrile (95:5, v/v). The standard mycotoxins and extracts were applied to TLC plates (Merck 5553, Merck KGaA, Darmstadt, Germany) and were resolved in a solvent system containing 8:1:1 (v/v/v) chloroform:acetone:2-propanol. The plates were developed by spraying with 20% aluminum chloride in ethanol and heating to 100 °C for 7 min. The presence of DON, NIV, 3-AcDON and 15-AcDON (Trilogy Analytical Laboratory Inc., Washington, MO) production was determined by visual comparison with known amounts of standards illuminated with UV light at 366 nm.<sup>31</sup>

### Data analyses

Correlations between variables were analyzed using the Pearson correlation coefficient. Fisher's protected LSD test was used for comparing the means of fungal propagules, mycotoxin levels and sample storage time. All analyses were conducted using Sigma STAT version 2.1 (Chicago).

## Results and discussion

Fungal contamination was observed in all of the wheat samples. The main fungi isolated were from the genera *Fusarium* (43%), *Aspergillus* (36%), *Alternaria* (33%), followed by *Sporothrix* (24%), *Eppicocum* (12%) and *Penicillium* (6%). There was no significant difference in the total fungal propagules ( $p > 0.05$ ) among the sampling periods, which indicates good processing practices. However, some genera, such as *Alternaria*, *Eppicocum* and *Fusarium*, occurred at higher frequencies in freshly harvested wheat and were significantly different from the remainder of the sampling period ( $p < 0.05$ ) (Fig. 1). On the other hand, *Aspergillus*, *Sporothrix* and *Penicillium*, which are known to cause spoilage of stored products, were present in high frequencies after 60 days of storage (Fig. 1).



**Fig. 1 – Isolation frequency (%) of fungal genera in silage samples; ■ – harvest; ■ – 60 days of storage; ■ – 90 days of storage; ■ – 120 days of storage.**

*Fusarium* spp. and *Aspergillus* spp., the main toxigenic molds, were the prevalent genera in wheat silage. These results are in agreement with several studies that found similar results in corn and sorghum silages.<sup>3,18,32–34</sup> Corn silages are heavily studied, but there are few data on mycoflora and mycotoxin contamination in wheat silages.

*F. graminearum* s.l., *F. acuminatum*, *F. poae*, *F. sambucinum*, *F. sporotrichioides*, *F. oxysporum*, *F. nygamai*, *F. lateritium*, *F. equiseti* and *F. subglutinans* were identified; *F. graminearum* s.l. was the prevalent species, with a relative density of 93%, which has been reported by other authors for wheat at the field stage.<sup>13,35–37</sup> These results differ from those found for corn and sorghum silages, in which *F. verticillioides* is the most frequent species.<sup>2,18,33,38</sup>

Within the genus *Aspergillus*, only *Aspergillus* section *Flavi*, *A. niger* aggregate and *A. clavatus* were identified, with *Aspergillus* section *Flavi* showing the highest relative density (98%) in according with its occurrence in corn and sorghum silages in Argentina and Brazil.<sup>5,18</sup> On the other hand, this result differs from results reported by another author who found high incidences of *A. fumigatus* in corn silages.<sup>39</sup> The absence of *A. fumigatus* in study samples decreases the dual hazards of ingestion of pathogenic spores and potential mycotoxin production.<sup>40</sup>

The isolates of *Sporothrix schenckii*-like morphogroup<sup>41</sup> are not commonly found in silages, but in this work, the isolate frequency was 23.8% and was only found in post-fermentation silage samples. This could be because *S. schenckii* is a dimorphic fungus that grows as a yeast (35–37 °C) and as mycelia (25–30 °C) in presence of CO<sub>2</sub>.<sup>42,43</sup> This species is an important human-pathogenic fungus and is the etiologic agent of sporotrichosis. The most common clinical presentation of this disease is the coetaneous form with or without regional lymphatic involvement. Sporotrichosis is considered to be a hazard among workers whose occupations bring them into frequent and sometimes traumatic contact with plant material or soil.<sup>44</sup>

*F. graminearum* s.l. was the most frequent isolate on recently harvested grains and grains stored up to day 60. However, *Aspergillus* section *Flavi* was recorded after 60 days of storage as showing a significant negative correlation with *F. graminearum* s.l. ( $r = -0.97$ ,  $p < 0.05$ ). *F. graminearum* s.l. exhibited a negative correlation with time of storage ( $r = -0.97$ ,  $p < 0.05$ ),

**Table 1 – Physical properties and mycotoxin levels in wheat silages during the storage time.**

Time (days)	$a_w \pm SD$	pH $\pm$ SD	MC% $\pm$ SD	Deoxynivalenol ( $\mu\text{g}/\text{kg}$ )		Aflatoxins ( $\mu\text{g}/\text{kg}$ )	
				Mean $\pm$ SD	Range	Mean $\pm$ SD	Range
60	0.693 $\pm$ 0.057	6.39 $\pm$ 0.5	11.7 $\pm$ 2.3	5300 $\pm$ 1691	3000–6800	3 $\pm$ 5	nd-11.9
90	0.672 $\pm$ 0.051	6.49 $\pm$ 0.30	12.4 $\pm$ 1.9	5525 $\pm$ 1499	3400–6800	12 $\pm$ 8	nd-18.1
120	0.709 $\pm$ 0.043	6.69 $\pm$ 0.30	14.6 $\pm$ 3.4	6007 $\pm$ 2122	3800–7900	17 $\pm$ 9	6–23

$a_w$ , water activity; SD, standard deviation; MC, grain moisture content; nd, not detected.

while *Aspergillus* section *Flavi* showed a positive correlation ( $r=0.99$ ,  $p<0.05$ ).

The study of pre-existing mycobiota in a given substrate can sometimes be used as a basis for estimating contaminating mycotoxins. Wheat grains are frequently contaminated simultaneously by several fungi, which are each able to produce several toxins.

Due to a lack of unified pre-existing methodologies for mycotoxin analysis in wheat silage samples, we chose the most appropriate available methods for these samples. Measured mycotoxin levels in wheat silage samples are shown in Table 1. The presence of mycotoxins was detected in all samples analyzed. The levels of aflatoxins ranged from 6.1 to 23.3  $\mu\text{g}/\text{kg}$ , whereas DON levels were between 3000  $\mu\text{g}/\text{kg}$  and 12,400  $\mu\text{g}/\text{kg}$ , with recently harvested samples showing higher and significantly different levels ( $p<0.05$ ). There were no significant differences in aflatoxin levels ( $p>0.05$ ) among the sampling periods.

Freshly harvested samples did not show aflatoxin contamination, but a high percentage of silo samples were positive for aflatoxins contamination, suggesting that *Aspergillus* section *Flavi* and aflatoxin contamination was enhanced during storage. However, only 16.7% of these showed levels exceeding the recommended limit for feed destined for dairy cattle and the maximum permissible level for human food in Uruguay (20  $\mu\text{g}/\text{kg}$ ).<sup>45,46</sup> Similar results were found in corn and sorghum silages from Argentina and Brazil,<sup>18,33</sup> while aflatoxin contamination was absent in wheat silages from Israel and the Netherlands,<sup>47,48</sup> probably due to the different climatic conditions in Uruguay.

As wheat grains arrive to silos contaminated with *F. graminearum* s.l., they could provide the initial DON levels presented in the silage. During the storage time (120 days), a reduction of 43% in DON contamination was detected. Conversely, in corn silages, DON levels increased during storage.<sup>33,49</sup> In this study, the reduction in the occurrence of DON could be considered an advantage of using silo bags.

Few studies have highlighted the importance of characterizing the toxicogenic profile of fungal species isolated from silages. *Aspergillus* section *Flavi* and *F. graminearum* s.l. were the dominant species, and various strains were able to produce aflatoxins and DON, respectively. The presence of toxicogenic strains in commodities constitutes a potential risk for animal health because the toxins can be produced in the substrate. This was confirmed by the presence of aflatoxins and DON contamination in silage samples. When evaluating aflatoxigenic capacity, 27.5% of *Aspergillus* section *Flavi* strains produced AFB1, 5% AFB2, 10% AFG1 and 17.5% AFG2. Trichothecene mycotoxin genotypes and chemotypes were

determined for isolates of *F. graminearum* s.l. The multiplex PCR assay demonstrated that all isolates were of the DON genotype. These assays demonstrated that all strains with the DON genotype were also of the 15-AcDON genotype. All strains with the 15-AcDON genotype produced 15-AcDON and DON as assessed by chemical analyses.

The physical properties of samples did not vary significantly during storage time ( $p>0.05$ ). Table 1 shows the physical properties of the silages. Water activities, pH and MC% ranged from 0.693  $a_w$  to 0.709  $a_w$ , from 6.39 to 6.69 and from 11.7 to 14.6, respectively. However, some samples had  $a_w$ , moisture content and pH levels that allowed fungal growth and mycotoxin production. Fungal growth generally occurs when the silo is not well packed, the water activity exceeded 0.7–0.8 and pH values ranged over 6.<sup>39,50,51</sup>

It is known that the minimal water activity necessary for fungal growth is below that required for the production of aflatoxins. As such,<sup>52</sup> Lacey et al. suggests 0.78 and 0.83 as the minimum water activity values that favor proliferation of *A. flavus* and the production of aflatoxins, respectively. In our case, although values were lower than those required, there was a large number of *Aspergillus* section *Flavi* isolates and high aflatoxin levels, probably due to oxygen presence or high temperatures. Some farmers in Uruguay remove silage for animal feeding by shoveling; this practice breaks compaction of the silo within the extraction area and favors aeration, thereby changing the redox potential.

On the other hand, *Fusarium* is not very adaptable to the anoxic and acidic environments of silo bags,<sup>5</sup> which could be the reason why *F. graminearum* s.l. was mainly present at harvest and could be an advantage of using this type of silo.

Wheat silages showed a high presence of *Aspergillus* section *Flavi* as well as high contamination of DON and aflatoxins, which are hazardous to human and animal health. The high fungal and mycotoxin levels present in silage could affect the palatability of feed and could cause animal disease or production losses. For dairy cattle, the problem does not end here because mycotoxins in feed can lead to production of metabolic products (AFM1) in dairy products that eventually affect human health, mainly that of infants. The adoption of regular screening for DON and aflatoxin levels in silages is recommended to avoid exposure of animals to contaminated feed and introduction of these compounds into the food chain. To date, no information exists about the presence of mycotoxins and their derivatives in milk from Uruguay. The results in the present study contribute to the sparse knowledge of mycobiota and contamination of mycotoxins in wheat silage. Further studies on the ecophysiological aspects of contaminant species would help to identify factors that need to be

controlled to diminish the development and production of mycotoxins in silo storage.

### Conflicts of interest

The authors declare no conflicts of interest.

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