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Research Note

How do Time, Tannin, and Moisture Content Influence Toxicogenic Fungal Populations during the Storage of Sorghum Grains?

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ABSTRACT

Cereal grains are usually ensiled to improve their nutritional value and are one of the main sources of feed for dairy cattle. However, during storage, grains can be contaminated with toxicogenic fungi. *Sorghum* is one of the most economically important cereals in the world. Therefore, the aim of this work was to evaluate the influence of storage duration and tannin and moisture content (MC) on toxicogenic fungal populations in sorghum grain storage. Samples that were prepared with varieties high in tannins (genotypes Morgan 108 and ACA 558, >5 g/kg dry matter) and with varieties low in tannin content (genotypes Flash 10 and ACA 546, <1 g/kg dry matter) were collected and manually compacted in experimental laboratory silos where they received different MC treatments: low (15 to 25%), medium (26 to 32%), and high (33 to 42%). Freshly harvested grains were analyzed at time 0, and stored grains were analyzed at 30, 90, and 180 days. Fungal isolation and identification were performed following conventional mycological methods. *Penicillium citrinum* (34%), *Aspergillus flavus* (60%), and *Fusarium nygamai* (68%) were the most abundant species. Rapid detection of aflatoxins and fumonisins in each sample was performed using enzyme-linked immunosorbent assay according to the AOAC method, and the quantification of aflatoxin B_1 was performed using high-performance liquid chromatography. In four samples of pre- and poststorage grains, aflatoxins were detected with levels of 6.7 to 28.8 μg/kg and aflatoxin B_1 with a level of 2 to 14 μg/kg. Fumonisins were only detected in two freshly harvested samples, with levels of 500 to 900 μg/kg. In general, storage time favored the increase of *Penicillium* populations and reduced *Aspergillus* and *Fusarium*. Conversely the abundance of the three populations was not affected by the MC. The results of this study show that fungal populations must be analyzed at different times.

HIGHLIGHTS

- Storage favored *Penicillium* colonization; fresh grains favored *Fusarium*.
- *Aspergillus* abundance in high-tannin grains decreased with storage time.
- Storage for 180 days inhibited fungal colonization in high- and low-tannin grains.
- Aflatoxins and fumonisin were found in few samples and at low levels in grains.

Key words: Moisture content; Mycotoxins; Storage time; Tannin content

Sorghum is the fifth most economically important cereal crop, with an average production over the last two decades of 60.2 Mt *(19)*. In Uruguay, over 10 years (2004-2005 to 2014-2015), production ranged from 84,700 to 238,000 tons *(20)*. In addition to its importance as a food, feed, and forage crop, sorghum also provides raw material for production of starch, fiber, dextrose syrup, biofuels, and alcohol, among other products *(35)*.

Thousands of years ago, ancient Egyptians were known to store harvested grains. In Uruguay, particularly, wet grain silage has played a preponderant role in the various dairy and livestock production systems of the country. In wet grain silage, harvested grain is conserved under anaerobic conditions with a humidity between 23 and 40% (optimum is 28%) without prior drying *(14)*. Because storage systems for seeds are artificial ecosystems, maintaining their quality and nutritional value is very important; fungal spoilage and mycotoxin contamination are among the greatest risks to stored feed, such as silage *(5)*. Fungal populations interact according to certain basic principles in ecology. Growth rate, frequency, and abundance of potential abilities to degrade seeds are related to the interaction of fungal and seed characteristics with the environment and the ecophysiological requirements for fungal growth *(55)*.

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Fungal growth on stored grains is an obvious sign of poor quality, indicating diminished sensory quality and nutritional value. Mycotoxins produced by grain fungi also pose a health hazard to animals and humans. Hence, grains must be safely stored after harvest at specific times of the year to ensure supply throughout the remainder of the year *(39)*. Microbial colonization of grains starts soon after grain emergence and may continue until the grain is utilized, but the major disturbance of the ecosystem occurs as the grain is harvested and placed in storage, with the change from the environmental conditions in the field to those in storage.

Several methods have recently been developed for efficient mass storage of grains. Environmental control via manipulation of ecological factors at storage facilities remains a basic and efficient measure to control fungal contamination in stored grains.

In addition to fungal spoilage, mycotoxin contamination is one of the greatest risks to stored feed, such as silage. The mycotoxins in stored grains are produced by three genera: *Aspergillus, Penicillium,* and *Fusarium (16, 23, 31)*. However, to date, there is no legislation to regulate the maximum acceptable content of mycotoxins in sorghum for commercial purposes, as there is for other cereals *(9)*.

García y Santos et al. *(24)* found that, for moist sorghum grains stored in silo bags, a long storage time of 180 days was beneficial for decreasing toxicogenic fungal populations, particularly on high-tannin grains. Consequently, the aim of this work was to study the effects of storage time, tannin level, and moisture content (MC) on the abundance of the main mycotoxigenic fungi that colonize freshly harvested and stored sorghum grains and to select the best conditions to reduce grain spoilage. Because mycotoxins in food and feed are one of the main food safety problems worldwide, this work also examines the occurrence of mycotoxins in freshly harvested and stored sorghum grains.

MATERIALS AND METHODS

Sampling procedure and silo grain treatments. Samples of *Sorghum* varieties with condensed tannins at low and high levels were collected immediately before the start of the ensiling process from five commercial dairy farms located in San José, Flores, and Canelones Departments (33°33′28.3′′ S 56°52′37.7′′ W, and 34°31′07.5′′ S 56°32′21.1′′ W) in south-central Uruguay. The rainfall during the *Sorghum* growing period (April to June) was 300 mm, an average precipitation for these months. At the time of harvest, grains had a MC of 26 to 32%.

Grain samples were collected from five farms selected in the previous study according to the variety of sorghum grains used for preparing the silages *(24)*. Three of the farms produced varieties with high tannin (HT) content (>5 g/kg dry matter [DM]). Of these, farm 1 produced genotype Morgan 108 (12.3 g/kg DM of tannin), and farms 2 and 3 produced ACA 558 (7.8 and 5.6 g/kg DM of tannin, respectively). The other two farms produced varieties with low tannin (LT) content (<1 g/kg DM). Of these, farm 4 produced genotype Flash 10 (0.9 g/kg DM of tannin), and farm 5 produced ACA 546 (0.7 g/kg DM of tannin).

From each of the five farms, 30 kg of grains was collected during the ensiling process. A 3-kg portion (moisture ranging from 26 to 32%) was homogenized and sampled for mycological analysis at time 0. The rest (27 kg) was used to develop experimental silos, under laboratory conditions. From each farm, nine experimental silos (observation units) were used for this study. In field conditions, farmers in Uruguay store wet sorghum grains in silo bags under MC that ranges widely, from 16 to 40% *(14)*. Fermentation problems have been detected under low and high MC, and we evaluated three MC levels to determine which was the best for grain storage: low (15 to 25), medium (26 to 32), and high (33 to 42%). For each farm and MC level, three experimental silos were developed. To evaluate storage time, one silo per farm and MC level was randomly assigned to be opened at either 30, 90, or 180 days. Then, a total of 50 samples (observation units) were analyzed: five samples of freshly harvested grains (three of HT and two of LT, one sample corresponding to each farm) and 45 experimental silos (one per storage time, MC, and farm). Experimental silos were round, white, 3-kg-capacity plastic containers (12 by 20 cm; polypropylene random copolymer, ATMA, La Paz, Uruguay), according to the methodology proposed by Aguerre et al. *(3)* and Brambillasca et al. *(12)*. Silos were hermetically closed and sealed. At each storage time (30, 90, and 180 days), they were weighed to evaluate whether MC had varied. If the variation was between 0 and 1%, modifications in MC were considered to be unnecessary. For mycological analysis, silos were opened and grains were randomly collected and afterward were discarded.

Chemical analyses. MC was determined using AOAC Official Method 925.09 *(7)*. A crucible was dried in an oven at 105°C for 1 h and placed in desiccators to cool. Condensed tannins were determined with the butanol-HCl method described by Makkar *(17, 38)*.

Mycological analysis. For fungal isolation, 100 grains of sorghum from each sample were randomly selected; after surface disinfection in a 0.4% solution of NaOCl for 1 min, they were rinsed three times with sterile distilled water and dried with sterile absorbent paper. Next, seeds were placed in Petri dishes containing potato dextrose agar (70139, Sigma-Aldrich, St. Louis, MO) and were incubated at 25°C for 7 to 10 days. At the end of this period, the resulting colonies were transferred to selective culture media for isolation: Czapek yeast extract agar supplemented with the antibiotics BC-RB for *Aspergillus* spp. *(15),* AFPA for *Aspergillus flavus* group *(50),* and CZID for *Fusarium* spp. *(2, 34, 58)*. The identification was performed according to the available guides for *Aspergillus* spp. *(30),* for *A. flavus* group *(50, 53),* for *Penicillium* spp. *(45, 46, 49, 51, 61, 64),* and for *Fusarium* spp. *(6, 13, 27, 29, 33, 44)*. The identification of isolates corresponding to *Fusarium* spp. and *Aspergillus* spp. was confirmed by means of molecular techniques as described by Mionetto *(42)*. *Fusarium* spp. were identified using the EF1 and EF2 specific primers, according to O'Donell et al. *(47)*. *Aspergillus* spp. were analyzed by means of ITS1 and ITS4, according to Vargas et al. *(62)*.

The isolation frequency and the relative abundance of genera or species were calculated according to McMullen and Stack *(40)* for each tannin content, MC, and storage time: relative frequency was the number of samples of occurrence per the total number of samples; relative abundance, the number of isolates of a fungal taxon per total number of isolates.

Analysis of mycotoxins in sorghum grains. Rapid detection of total aflatoxins from each sample was performed in triplicate using enzyme-linked immunosorbent assay (ELISA) with the Ridascreen Fast Aflatoxin SC (R-Biopharm AG, Darmstadt, Germany) commercial kit, according to the AOAC method *(8)*.

Approximately 2 g of each sample was disintegrated, weighed, and extracted with 10 mL of a methanol-water mixture (7+3) to separate aflatoxins. Sample extract (100 µL) and horseradish peroxidase enzyme conjugate (100 µL, diluted in 1% bovine serum albumin [BSA] in phosphate-buffered saline [PBS]) were added to the antibody-coated wells, and the mixed solution was incubated for 60 min. After washing with 0.1% Tween 20 in PBS, substrate-chromogen solution (3,3′,5,5′-tetramethylbenzidine–hydrogen peroxide in acetate buffer, pH 5.5, 100 µL) was added to all testing wells, and the plate was incubated for 30 min. The plate was read in a dual wavelength mode (450/650 nm) after stopping the color development with 1.25 M sulfuric acid (50 µL). For the control and blank wells, 16% methanol and diluted sample extract were used for their respective standard curves. The final absorbance was calculated by subtracting the absorbance of the corresponding blank wells (background color). The limit of detection of aflatoxins (ELISA) was 2 µg/kg, and the range of detection was 2 to 100 µg/kg.

Aflatoxin B_1 (AFB_1) standard or undiluted sample extract (50 µL) and horseradish peroxidase enzyme conjugate (100 µL, diluted in 1% BSA in PBS) (in a direct assay) or a coating antigen (in an indirect assay) were prepared from a hapten that is structurally similar but not identical to that used for antibody production. Detection and quantification of AFB_1 from each sample was performed in triplicate using high-performance liquid chromatography according to the methodology proposed by Trucksess et al. *(61)*. Twenty-five grams of sample and 100 mL of acetonitrile:water (84:16, v/v) were placed in a high-speed blender for 3 min. The mixture was filtered through Whatman No. 4 filter paper, and a 5-mL aliquot of this filtrate was passed through a MycoSep 224 cleaning column (Romer Labs Inc., Union, MO). Of this extract, 2 mL was brought to dryness and then resuspended in 400 µL of water:methanol:acetonitrile at (4:1:1, v/v). An aliquot (200 µL) was derivatized with 700 µL of trifluoroacetic acid:acetic acid:water (20:10:70, v/v). Chromatographic separations were performed on a reverse-phase column (150 by 4.6 mm id., 5-µm particle size; silica gel, Varian, Inc., Palo Alto, CA). Methanol:water (60:40, v/v) was used as the mobile phase at a flow rate of 1 mL min^{-1}. The fluorescence of aflatoxin derivatives was recorded at λ 360 nm and λ 460 nm. Standard curves were constructed with different levels of AFB_1. This toxin was quantified by correlating the peak heights of the sample extracts with those of standard curves. The recovery of AFB_1 ranged from 73 to 98%, the range of detection from 0.02 to 2 µg/kg, and the range of quantification from 0.05 to 5 µg/kg. The limit of quantification of AFB_1 was 0.05 µg/kg *(21)*. The detection limit of the analytical method was 0.4 µg/kg *(48)*.

Fumonisin analysis was performed using ELISA with the Ridascreen Fast Fumonisins (R-Biopharm AG, Darmstadt, Germany) commercial kit. In triplicate, 5 g of ground sample was weighed and extracted with 25 mL of methanol-water mixture (7+3) to separate fumonisins. The sample extract was shaken vigorously for 3 min with a shaker, filtered through Whatman No. 1 filter paper and diluted 1:14 (1+13) with distilled water. Then, 50 µL was added to each microtiter well with equal volumes of enzyme conjugate and anti-fumonisin antibody solution, and the mixture was incubated for 10 min. After washing, 100 µL of substrate-chromogen solution was added to the well, mixed, and incubated for 5 min in the dark. The reaction was stopped with 100 µL of 1 N sulfuric acid. The detection limit for fumonisins (ELISA) was 0.222 mg/kg, and the range of detection was 0.222 to 6 mg/kg, according to the manufacturer's specifications.

TABLE 1. *Statistical analysis of the relative frequencies of* Penicillium *spp.*, Aspergillus *spp., and* Fusarium *spp. from freshly harvested and ensiled sorghum grains*[a]

	Penicillium							*Aspergillus*							*Fusarium*						
	High tannin				Low tannin			High tannin				Low tannin			High tannin				Low tannin		
Variable	*N*	*n* (%), Ab *N* = 7	*n* (%), Pr *N* = 29	*P*	*N*	*n* (%), Ab *N* = 1	*n* (%), Pr *N* = 23	*P*	*N*	*n* (%), Ab *N* = 11	*n* (%), Pr *N* = 25	*P*	*N*	*n* (%), Ab *N* = 4	*n* (%), Pr *N* = 20	*N*	*n* (%), Ab *N* = 26	*n* (%), Pr *N* = 10	*P*	*N*	*n* (%), Ab *N* = 19	*n* (%), Pr *N* = 5	*P*
MC (%)	36			>0.9	24			>0.9	36			0.3	24			36			0.5	24			>0.9
High		3 (43)	9 (31)			0 (0)	8 (35)			3 (27)	9 (36)			2 (50)	6 (30)		10 (38)	2 (20)			7 (37)	1 (20)	
Medium		2 (29)	10 (34)			1 (100)	7 (30)			6 (55)	6 (24)			1 (25)	7 (35)		9 (35)	3 (30)			6 (32)	2 (40)	
Low		2 (29)	10 (34)			0 (0)	8 (35)			2 (18)	10 (40)			1 (25)	7 (35)		7 (27)	5 (50)			6 (32)	2 (40)	
Time (days)	36			0.4	24			>0.9	36			0.06	24						**0.04**	24			0.11
0		3 (43)	6 (21)			0 (0)	6 (26)			0 (0)	9 (36)			0 (0)	6 (30)		3 (12)	6 (60)			3 (16)	3 (60)	
30		0 (0)	9 (31)			1 (100)	5 (22)			2 (18)	7 (28)			0 (0)	6 (30)		7 (27)	2 (20)			4 (21)	2 (40)	
90		2 (29)	7 (24)			0 (0)	6 (26)			4 (36)	5 (20)			2 (50)	4 (20)		8 (31)	1 (10)			6 (32)	0 (0)	
180		2 (29)	7 (24)			0 (0)	6 (26)			5 (45)	4 (16)			2 (50)	4 (20)		8 (31)	1 (10)			6 (32)	0 (0)	

[a] Statistical test performed: chi-square test of independence; Fisher's exact test; N (%); Ab, absence; Pr, presence; MC, moisture content. The bold number indicates a significant difference.

TABLE 2. *Analysis of the relative abundance of* Penicillium *spp.,* Aspergillus *spp., and* Fusarium *spp. from two varieties of freshly harvested and ensiled sorghum grains*[a]

	Penicillium			*Aspergillus*			*Fusarium*		
Variable	High MC	Medium MC	Low MC	High MC	Medium MC	Low MC	High MC	Medium MC	Low MC
High tannin									
0 days	—	0.11	—	—	0.29	—	—	0.29	—
30 days	0.23	0.88	0.2	0.42	0.03	0.28	0	0	0.03
90 days	0.55	0.73	0.23	0.05	0.09	0.3	0	0	0.03
180 days	0.42	0.58	0.39	0.05	0.08	0.19	0	0.02	0.03
Low tannin									
0 days	—	0.04	—	—	0.01	—	—	0.14	—
30 days	0.37	0.02	0.04	0.21	0.08	0.04	0	0.05	0.13
90 days	0.62	0.53	0.18	0.15	0.12	0.35	0	0	0
180 days	0.79	0.47	0.47	0.14	0.13	0.07	0	0	0

[a] Freshly harvested, time 0; MC, moisture content (%).

Statistical analysis. The relative frequency of *Aspergillus, Fusarium,* and *Penicillium* isolates was evaluated by chi-square and Fisher's analysis. The relative abundance of fungi and mycotoxins was determined by the logistic regression model to evaluate the association between the study variables. Split-plot designs were analyzed for each grain variety (HT or LT) using the generalized linear mixed model in R *(54)*. Interactions between MC and storage time were included as a fixed effect. MC (high, medium, and low) was considered by main plot and the fresh harvest (time 0) and storage time (30, 90, and 180 days) were considered by split plot. Significance was declared at $P < 0.05$.

RESULTS

The presence of fungi was observed in 49 of 50 sorghum samples. The most frequent fungi isolated in freshly harvested and stored samples corresponded to *Penicillium* spp. (88%), *Aspergillus* spp. (70%), and *Fusarium* spp. (18%).

As shown in Table 1, the frequencies of all fungi increased after grains were freshly harvested (time 0) up to 30 days of storage and then gradually decreased over time until the end of the period (day 180); only *Fusarium* showed a statistically significant difference ($P = 0.04$) (Table 1).

In freshly harvested and storage samples, the distribution of species within each genus showed that one species was dominant and several were rare. *A. flavus* (60%) was the most abundant species of this genus, and *A. fumigatus* (14%), *A. niger* (14%), *A. candidus* (6.5%), and *A. clavatus* (3.5%) were present with lower abundance and as rare species, along with *A. ochraceus* (1.1%) and *A. niveus* (0.6%). In *Fusarium, F. nygamai* (68%) was the most abundant; *F. graminearum* was less abundant (26.4%); and *F. tricintum* (2.8%), *F. oxysporum* (1.4%), and *F. semitectum* (1.4%) were rare species. Of *Penicillium* isolates, *Penicillium citrinum* (34%) was the most abundant species of this genus present in all samples, and *P. verrucosum* (4%) was present in a few samples with low abundance.

In HT and LT fresh grain samples (time 0), the relative abundance of *Penicillium* spp. was significantly lower ($P < 0.01$) than in stored grains at 30, 90, and 180 days with the three MCs (Table 2), as shown in Table 3. But only the relative abundance of LT grains with medium and low MC evidenced a statistically significant difference in relation with the grains with high MC (Table 3). If the interactions of MC and time are considered, a statistically significant difference in relative abundance can be observed in HT grains with medium MC at 30 days and with low MC at 90 days and in LT grains with medium MC at 30 days and with low MC at 30 and 180 days ($P < 0.01$).

The relative abundance of *Aspergillus* spp. in HT fresh samples (time 0) was higher than in stored samples with the three MC levels, except at 30 days with high MC (Table 2), without evidence of significant difference (Table 3). In LT fresh samples (time 0), the relative abundance of *Aspergillus* was lower than at 30, 90, and 180 days of storage with high and medium MC. However, in grains with low MC at 180 days, *Aspergillus* abundance was slightly higher than in freshly harvested grains (time 0), without significant difference. However, the effect of storage time evidenced a significant difference ($P < 0.01$) in the relative abundance of *Aspergillus* present in grains of both varieties. The effect of MC levels on *Aspergillus* spp. abundance was not statistically significant (Table 3). However, if interactions of MC and time are considered, a statistically significant difference in relative abundance can be observed only in HT grains with medium MC at 30 day ($P < 0.01$) (Table 4).

The relative abundance of *Fusarium* spp. in HT fresh grains (time 0) was significantly higher ($P < 0.01$) than in storage grains at 30, 90, and 180 days (Table 2), as shown in Table 3. Conversely, the effect of MC levels on the relative abundance was not statistically significant (Table 3). Moisture and time interactions did not occur.

Total aflatoxins were detected in only 4 (8%) of the 50 samples, one from a freshly harvested sample and three from stored samples. In the freshly harvested (time 0) HT sample, 6.7 μg/kg total aflatoxins and 2 μg/kg of AFB_1 were detected. Total aflatoxins detected in the three stored samples were as follows: (i) 20 μg/kg in a LT sample with high MC at day 90 (AFB_1 was not detected); (ii) 14 μg/kg in a LT sample with medium MC at day 180 (14 μg/kg of AFB_1 was detected); (iii) 28.8 μg/kg in a HT sample with low MC at day 180 (AFB_1 was not detected).

Fumonisin was detected only in two freshly harvested samples contaminated with *Fusarium,* with levels ranging

TABLE 3. *Statistical analysis of the relative abundance of* Penicillium *spp.,* Aspergillus *spp., and* Fusarium *spp. from two varieties of freshly harvested and ensiled sorghum grains*[a]

	Penicillium						*Aspergillus*						*Fusarium*					
	High tannin			Low tannin			High tannin			Low tannin			High tannin			Low tannin		
Variable	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
MC (%)																		
High	—	—		—	—		—	—		—	—		—	—		—	—	
Medium	2.04	0.70, 5.97	0.2	0.22	0.09, 0.56	<0.01	0.70	0.48, 1.02	0.06	0.44	0.16, 1.19	0.11	0.99	0.51, 1.91	>0.9	1.25	0.70, 2.21	0.4
Low	0.52	0.18, 1.51	0.2	0.23	0.09, 0.58	<0.01	1.18	0.84, 1.67	0.3	0.55	0.21, 1.44	0.2	1.36	0.74, 2.50	0.3	1.25	0.72, 2.15	0.4
Time (days)																		
0	—	—		—	—		—	—		—	—		—	—		—	—	
30	9.34	6.33, 13.8	<0.01	2.35	1.21, 4.59	<0.01	0.39	0.28, 0.53	<0.01	6.35	2.34, 17.2	<0.01	0.24	0.11, 0.53	<0.01	0.83	0.50, 1.38	0.5
90	14.9	10.0, 22.1	<0.01	17.7	7.81, 40.0	<0.01	0.52	0.37, 0.75	<0.01	6.7	5.35, 52.4	<0.01	0.14	0.05, 0.34	<0.01	0.00	0.00, Inf	>0.9
180	13.3	8.31, 21.2	<0.01	26.8	13.1, 55.1	<0.01	0.30	0.17, 0.51	<0.01	5.52	1.67, 18.2	<0.01	0.07	0.01, 0.50	<0.01	0.00	0.00, Inf	>0.9

[a] OR, odds ratio; CI, confidence interval; MC, moisture content (%). Numbers in bold indicate significance differences.

from 500 μg/kg in HT grains to 900 μg/kg in LT grains; it was not detected in stored samples.

DISCUSSION

The abundance of the most important toxicogenic fungi was significantly different between freshly harvested and stored grains with different MC levels and storage times. MC affected only the *Penicillium* population in LT grains: *Penicillium* levels were lower in grains with medium and low MC levels compared to grains with high MC. On the other hand, storage time favored the increase of *Penicillium* populations but reduced *Aspergillus* and *Fusarium* populations. A previous study found that, for moist sorghum grains stored in silo bags, 180 days of storage led to decreases in populations of the three toxicogenic fungi, particularly on HT grains *(24)*.

The main fungal contamination of sorghum grains found in this study was qualitatively comparable to that found in other sorghum-producing countries worldwide *(33)*. However, no similar quantitative comparison could be established because studies investigating fungal contaminants of stored sorghum grains for a long period (180 days) are not frequently performed. Wheat grains stored for 6 months showed the presence of *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp., as recorded here *(11, 52)*. These fungi were associated with cereal seeds and growing plants from the time the seed was sown as part of the agroecosystem to the time the new seeds were harvested, transported, and stored as part of a new grain ecosystem *(55)*.

Fungal contamination varies in occurrence and magnitude depending on the conditions under which a crop is grown, harvested, and stored *(47)*. In the sorghum grains analyzed here, *Penicillium* was favored in HT and LT grains under storage conditions, and *Aspergillus* was favored more in HT than in LT freshly harvested grains. Hence, it is important to take into account the amount of precipitation in the crop season to select the MC of sorghum grains for storage *(25)*.

The infrequent contamination and low levels of aflatoxins found in the freshly harvested grain samples could probably be related to rainfall values during the growing period. In stored grains, aflatoxins were absent or were detected at low levels in few samples; they were lowest in samples with medium MC, similar to fresh grain samples, and highest in samples with low or high MC. Conversely, Magan and Aldred *(36),* under silage conditions, found that grains with low water content were critical for safe storage. On the other hand, under laboratory conditions, Montani et al. *(43)* showed that medium MC was the most favorable condition for AFB_1 production in corn grains for all incubation times; the equivalence was approximately calculated according to values from Magan et al. *(37)*. Lahouar et al. *(32)* found that AFB_1 production could be avoided by storing sorghum grains with low MC and that the highest levels were produced with higher MC. Weledesemayat et al. *(65)* detected AFB_1 (2.26 μg/kg) in sorghum grains with low MC. Comparison of the MC percentage of sorghum grains with the water activity of other grains was approximately calculated according to

TABLE 4. *Statistical analysis of the interactions between moisture content and storage time on the relative abundance of* Penicillium *spp. and* Aspergillus *spp. of two varieties of sorghum grains*[a]

	Penicillium						*Aspergillus*					
	HT			LT			HT			LT		
MC × time	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
Medium MC × 30 days	31.5	8.99, 110	**<0.01**	0.02	0.00, 0.15	**<0.01**	0.04	0.01, 0.16	**<0.01**	0.22	0.02, 2.02	0.2
Low MC × 30 days	1.26	0.45, 3.54	0.7	0.12	0.03, 0.53	**<0.01**	0.49	0.21, 1.12	0.09	0.24	0.02, 2.31	0.2
Medium MC × 90 days	3.03	0.91, 10.0	0.07	0.64				0.82, 12.9	0.09	0.69	0.04, 12.7	0.8
Low MC × 90 days	0.12	0.05, 0.34	**<0.01**	0.17				0.10, 4.15	0.6	3.24	0.31, 73.3	0.3
Medium MC × 180 days	3.02	0.71, 12.8	0.13	0.22	0.04, 1.30	0.10	2.40	0.02, 1.32	0.09	3.31	0.06, 14.4	>0.9
Low MC × 180 days	0.70	0.18, 2.64	0.6	0.15	0.03, 0.76	**<0.01**	5.40	0.59, 49.2	0.13	0.31	0.02, 4.55	0.4

[a] OR, odds ratio; CI, confidence interval; HT, high tannin; LT, low tannin; MC, moisture content (%). Numbers in bold indicate significance differences.

values from Magan et al. *(37)*. These conflicting results likely stem from different conditions during the sorghum growing period and at the time of sampling.

Uruguay follows the limits determined by the common legislation of countries that constitute Mercosur; the maximum limit of aflatoxins allowed is 20 μg/kg for corn destined for dairy cow consumption *(41)*. Moreover, the Ministry of Livestock, Agriculture, and Fisheries of Uruguay recommends a maximum limit of 5 μg/kg for AFB_1 in diets intended for dairy cows, as indicated by European Union regulations *(22)*. Because stored grains are one of the main sources for dairy cow nutrition in Uruguay, there is a risk of aflatoxicosis, which has severe clinical symptoms and which reduces milk yield and feed conversion *(57, 59, 63)*. The most important impact on human health is due to the metabolic transformation of AFB_1 to AFM_1, present in milk *(56, 60)*.

Fumonisins produced by isolates of *F. nygamai* were only detected in two freshly harvested samples of HT and LT grains, corresponding to the scarce presence or absence of *F. nygamai* in silage grains. According to Da Silva et al. *(18),* the levels of aflatoxins and fumonisins detected in freshly harvested and silage sorghum grains depend on the prevailing abiotic factors (MC, temperature, relative humidity, and mean rainfall) during the sorghum growth period and at the time of sampling.

It seems probable that, apart from environmental conditions during preharvest and storage of grains, tannin content may also influence fungi abundance during storage. Tannins are recalcitrant resources for biodegradation, mainly due to the ability of these molecules to inhibit microbial growth by binding strongly to proteins and polysaccharides such as cellulose and pectin *(10)*. Condensed tannins are more resistant to microbial decomposition, whereas hydrolysable tannins are more easily degraded by some microorganisms *(17)*.

On the other hand, *Aspergillus* and *Penicillium* species are the most active microorganisms capable of producing tannase *(1)*. In animal feed, tannase is used to reduce the antinutritional effects of tannins and to improve digestibility, an activity that is accomplished by these fungi *(4, 26, 43)*.

Although the results of this study have shown that few samples were contaminated and mycotoxin levels were low, sorghum silage must be periodically monitored to avoid animal production impairment and hazards to animal and human health *(28)*. In addition, because sorghum grains are an important resource in Uruguay, legislation is needed to regulate the maximum acceptable limits of mycotoxins in these grains used for commercial purposes *(9)*.

Briefly, the results of this study showed that stored grain was more favorable to *Penicillium* spp. colonization than fresh grain (time 0); in contrast, after 30 days of silage, the relative abundance of *Fusarium* spp. decreased. Also, the abundance of *Aspergillus* spp. in HT grains decreased with storage time. Low toxicogenic fungal contamination and low levels of mycotoxin were detected, which suggests that periodic monitoring of sorghum silage must be performed at different times according to fungal population evolution and mycotoxin production.

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