Occurrence of fungi and mycotoxins in corn silage, Jalisco State, Mexico

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Summary

The aim of this study was to evaluate the chemical composition, mold count and mycotoxin contamination of corn silage collected during a six month-period. The results indicated that the chemical composition and the physicochemical parameters evaluated did not show significant variation during the sampling time. Fungal count on RBC ranged from 1.7×10^3 to 9×10^8 CFU/g. *Mucor*, *Penicillium* and *Aspergillus* spp. were the most frequent fungal species in the corn silage. *Fusarium* count ranged from 1.6×10^3 to 1.6×10^8 CFU/g in Nash Snyder culture media. Aflatoxin B, fumonisins, ochratoxin A, ochratoxin B, deoxynivalenol, and zearalenone were detected throughout the period of corn silage maintenance (100% positive samples). However, only deoxynivalenol levels were higher than the maximum limit recommended by the FDA.

Key words   Mycotoxins, Fungi, Corn silage

Incidencia de hongos y micotoxinas en el ensilaje de maíz en el estado de Jalisco, México

Resumen

El propósito del estudio fue evaluar la composición nutricional y la contaminación por hongos y micotoxinas en el ensilado de maíz. Se recogieron muestras de ensilado durante seis meses. Los resultados obtenidos mostraron que los parámetros físico-químicos del ensilaje se mantuvieron sin variaciones indicando la correcta conservación del alimento. El recuento fúngico general sobre RBC se presentó en el rango de $1,7 \times 10^3$ a 9×10^8 UFC/g, siendo los principales géneros *Mucor*, *Penicillium* y *Aspergillus*. El género *Fusarium* presentó un recuento de $1,6 \times 10^3$ a $1,6 \times 10^8$ UFC/g. La presencia de micotoxinas pudo observarse durante todo el periodo de conservación del ensilado de maíz (100% de muestras positivas). Durante agosto y septiembre, la micotoxina desoxinivalenol (DON) presentó niveles de 5,3 y 6,7 ppm, respectivamente, diferentes estadísticamente ($p < 0,05$) en comparación con otros meses y superiores al límite máximo recomendado por la FDA. El resto de las micotoxinas no presentaron diferencias significativas entre los diferentes meses, siendo sus niveles menores al límite establecido. Los resultados demuestran la simultaneidad de presentación de las principales micotoxinas en el ensilado de maíz asociadas con efectos adversos en la salud animal.

Palabras clave   Micotoxinas, Hongos, Ensilado de maíz

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Ensiling is a forage preservation method which allows the storage of forages for its later use with minimum losses in the nutritional quality. In the process, the lactic acid bacteria ferment the water soluble carbohydrates into lactic acid and acetic acid [12]. These two acids decrease the pH value and thus the spoilage microorganisms are inhibited [22]. However, several factors such as insufficient drying, condensation, moisture content, heat, insects, and other conditions could lead to undesirable growth of fungi that can cause aerobic spoilage [6].

Fungal growth can reduce the nutritional value of corn silage. Also, there is a possibility for production and accumulation of mycotoxins [16]. The dominant genera reported in corn silage include *Aspergillus*, *Penicillium*, *Fusarium*, *Mucor*, *Absidia*, *Monoascus*, *Scopulariopsis*

and *Trichoderma* [4,6,22]. Mycotoxins are biologically active compounds with acute and chronic impact on human and animal health. The production of toxins could occur during culture, harvest or storage of grains and forage associated with favorable environmental conditions (high temperature, moisture content and high relative humidity) [24]. Food and feed contamination by mycotoxins have been recently characterized by the World Health Organization (WHO) as chemical hazard [5]. The most significant mycotoxins are aflatoxins (AFT), ochratoxins A and B (OT), deoxynivalenol (DON), zearalenone (ZEN), T-2 toxin and fumonisins (FBs).

The objectives of this study were to determine *(i)* the occurrence of fungi during the corn silage *(ii)* the mycotoxins contamination, and *(iii)* chemical composition of corn silage.

Materials and methods

Samples. Whole-plant corn white hybrid was harvested, mechanically chopped approximately at 30% of DM, and cut to a theoretical length of 0.95 cm. Silages were realized using a concrete bunker silo sealed with a plastic cap. The silage had been stored for 2 months before the sampling began. A total of 36 corn silage samples were collected from one silo located in Acatic County, Jalisco State in 2006. Sampling periods comprised January, February, March, August, September, and October. The sampling schedule was according to the feed requirements for a dairy farm. When the silo was opened, the first sample was taken to evaluate the initial quality of silage. The last three months were included to evaluate the effect of post-season rain in the preservation and quality of the silage. Each month, two samples were taken at three different levels (at 1, 2, and 3 m from the silo floor) and 30 cm depth. The samples were hand collected and the composites samples (5 kg) were homogenized and reduced to obtain three sub-samples of 1 kg for analytical determinations.

Chemical analyses and lactic acid content in corn silage. Corn silage samples were analyzed for moisture (M%), crude protein (CP%), crude fat (CF%), fiber (F%), ash (A), neutral detergent fiber (NDF), and acid detergent fiber (ADF) according to 930.36, 954.04, 954.02, 962.09, 942.05, and 973.18 AOAC methods [9], respectively. Nitrogen-free extract (NFE) was calculated by difference. Lactic acid (LA) was evaluated according to the procedures of Minson and McLeod [18]. The pH was measured according to the AOAC TMECC 0.4.11A. The dry matter (DM) content was determined by heating material at 65 °C for 18 h.

Isolation and identification of fungi. Five grams of each sample were mechanically homogenized in peptone water (495 ml, 0.1%) for 1 min. Tenfold serial dilution was performed up to 10^{-6} in peptone water (0.1%). RBC plates were incubated at 25 ± 1 °C for 7 days. The Nash-Snyder plates were incubated at 24 ± 1 °C for 7 days. After incubation, the enumeration of fungal colonies was carried out and expressed as colony forming units per g of corn silage (CFU/g). For fungal identification at genus level, each isolated colony was microscopically observed and their physical morphological characteristics were compared to the keys of Samson et al. [23] and Nelson et al. [21].

Mycotoxin analyses. The quantitative analyses of mycotoxins in samples were performed by competitive ELISA using the AgraQuant® test kit (Romer Labs). The protocols for extraction and quantification were done according to the indications of the manufacturer. The correlation coefficients of the calibration curves for AFT, FBs, OT, ZEN, and DON were 0.991, 0.998, 0.999, 0.969, and 0.973, respectively.

Statistical analyses. Analyses were made using Sigma STAT program version 2.01 (Chicago). To establish the significant differences, Tukey´s test ($p < 0.05$) was performed.

Results and discusion

Results of the chemical composition are summarized in table 1. The pH-value was between 3.48 and 3.97 (mean = 3.71). Previous studies have shown an inhibition on microorganism growth and the preservation of corn silage at low pH values (around pH 4) [3]. In general, the crude protein ranged from 8.98 to 12.93% and presented little changes. The main variation occurred in samples from August and September. Hunt et al. [10] reported the effect of different maturity stage on the chemical composition of corn silage. In their studies, the moisture content (%) was 54.6-68.3%. This is an important factor in order to assure the quality of the silage. Other researchers agree that the optimal moisture content is from 65-70%. In our study, the moisture content ranged from 71.25 to 79.88% (mean 76.48%). This was higher than the recommended; however, the nutritional quality was not affected. There was not statistical difference among sampling period for the chemical components analyzed ($p > 0.05$).

Total fungal colony count on RBC culture media ranged from 1.7×10^3 to 9×10^8 CFU/g (Figure). The most frequent fungi detected and recovered from corn silage samples were *Mucor* spp. (29.2%), *Penicillium* spp. (17.5%), and *Aspergillus* spp. (8.3%). Other genera found

Table 1. Chemical composition of corn silage (%) during preservation stage.

	January	February	March	August	September	October	Mean ± SD
pH	3.88	3.91	3.97	3.48	3.54	3.50	3.71 ± 0.23
M%	77.94	79.35	79.88	75.7	71.25	74.80	76.49 ± 3.25
DM%	22.05	20.65	20.12	24.3	28.75	25.20	23.51 ± 3.25
CP%	8.98	9.42	9.17	12.14	12.93	9.43	10.34 ± 1.72
F%	30.94	33.14	33.61	41.25	37.53	29.93	34.40 ± 4.26
CF%	1.81	1.85	1.73	0.85	1.03	1.54	1.47 ± 0.43
NFE%	48.01	46.42	47.67	36.24	38.39	51.56	44.72 ± 6.02
A%	6.92	7.57	7.04	9.52	10.12	7.44	8.10 ± 1.37
NDF%	62.81	61.16	66.34	66.5	62.97	68.95	64.79 ± 2.93
ADF%	42.91	44.02	47.78	45.61	45.66	44.61	45.10 ± 1.67
LA%	6.62	5.61	5.65	5.69	5.27	5.38	5.70 ± 0.48

Moisture (M), dry matter (DM), crude protein (CP), crude fat (CF), crude fiber (F), nitrogen-free extract (NFE), ash (A), neutral detergent fiber (NDF), acid detergent fiber (ADF), lactic acid (LA). SD= Standard deviation.

Table 2. Total levels of aflatoxins (AFT), fumonisins (FBs), ochratoxins (OT), deoxynivalenol (DON), and zearalenone (ZEN) in corn silage.

	AFT (ppb)		FBs (ppm)		OT (ppb)		DON (ppm)		ZEN (ppb)	
	Mean ± SD		Mean ± SD		Mean ± SD		Mean ± SD		Mean ± SD	
January	15.2	2.0	0.7	0.3	5.3	0.4	1.4[a]	0.9	343.6	194.6
February	15.7	0.9	0.1	0.1	5.5	0.5	1.4[a]	0.6	421.2	120.9
March	14.8	0.7	0.4	0.004	5.8	0.6	1.5[a]	0.3	482.1	65.4
August	12.5	1.5	0.4	–	5.2	0.7	5.3[b]	0.3	193.7	26.0
September	12.9	1.2	0.4	0.1	4.7	0.6	6.7[b]	0.6	168.8	24.8
October	14.2	0.4	0.6	0.3	4.4	0.1	1.9[a]	0.4	384.9	29.3

Limits of detection: AFT=3 ppb; FBs=0.2 ppm; OT=2 ppb; DON=0.2 ppm; ZEN=10 ppb; SD= standard deviation; ppb= parts per billion; ppm= parts per million.
Different superscript letters on each column indicate significant difference according to Tukey´s test (p < 0.05).
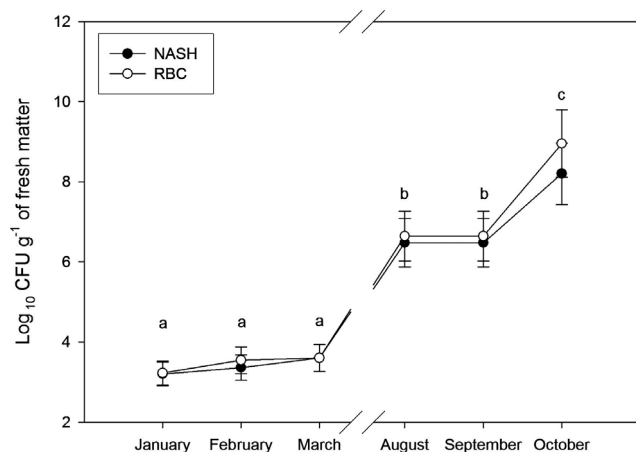

Figure. General count of fungi (RBC) and count of species of *Fusarium* (Nash Snyder) in corn silage. Different letters indicate significant difference according to Tukey´s test (p < 0.05).

with low incidence were *Fusarium* spp., *Alternaria* spp., *Geotrichum* spp., and micelia sterilia in culture media. *Fusarium* spp. count ranged from 1.6×10^3 to 1.6×10^8 CFU/g in Nash Snayder culture media (Figure). The first three sampling periods (January, February, and March) showed not statistical differences, which indicate good processing practices. Samples from August to October were visibly contaminated with fungi and the statistical analysis on the count indicated significant differences (p< 0.05). In this region of Mexico, the rainfall season occurs from June to September; therefore, the higher count of fungi could also be due to increases of relative humidity in the environment.

When the silo is opened for feeding-out, the air access increases the possibility of contamination by bacteria; thus, the yeasts and moulds can initiate an aerobic deterioration process [1]. Therefore, reduction in the quality of silage and mycotoxins production may occur. In a 1.5 m silage depth, the products of lactic acid fermentation and the aerobic conditions have been reported that exerted a mycostatic effect [13]. This finding agrees with studies by Krustov and Kristov [13] and El-Shanawany et al. [4]. The increase in fungal count during the last sampling period indicated a loss of feed safety. This has been reported when the fungal populations are greater than 10^4 CFU/g. Moon et al. (19) and Bolsen et al. (2) reported that yeasts and moulds counts increased to 10^6 CFU/g in corn silage after four days of fermentation.

The mycotoxin levels in the corn silage samples are shown in table 2. The presence of mycotoxins was detected in all the samples analyzed. The levels of total aflatoxin (AFT) ranged from 12.5 to 15.7 ppb, whereas the fumonisin (FBs) levels were below of 0.7 ppm. The results

for ochratoxin (OT) ranged from 4.4 to 5.8 ppb and zearalenone (ZEN) from 168.8 to 482.1 ppb. Deoxynivalenol (DON) levels ranged from 1.4 to 6.7 ppm, being samples from August and September those with the higher levels and were statistically different from the remainder. There were no significant differences in mycotoxins levels for AFT, FBs, OT, and ZEN (p > 0.05) among the sampling period.

According to the regulations of mycotoxins proposed by the US Food and Drug Administration (FDA), the action levels are set at no more than 20 and 100 ppb of aflatoxins in feed destined to dairy and beef cattle, respectively. The advisory limits for fumonisins (FBs) and deoxynivalenol (DON) were set to 50 ppm and 5 ppm, respectively. For ochratoxin (OT) and zearalenone (ZEN) no regulatory limits have been established by the FDA; however, it is recommended that ZEN not exceeds 300 ppb for dairy cattle feed. Our results showed that it is possible to detect AFT, FBs, OT, DON, and ZEN presence at different levels in the corn silage. Nedělník and Moravcová [20] reported that mycotoxins in silages are produced during the vegetative growth, harvest and storage phases. In our work, the low concentration of mycotoxins detected in the silage can not be considered as a risk for the animal health. However, there is the possibility of a synergic effect between toxins. Studies have reported the synergism between DON/FB1 [15], FB1/Toxin T-2 [14] and AFB1/FB1 [8]. In dairy cattle the mycotoxins have been shown to have negative effects on milk production and the immunological system of the animals [11].

Other studies have shown that during the preservation of silage, no significant changes in the concentration of ZEN, DON [16], and AFB1 [17] have been detected. Also, a study in Czech Republic (2002-2003), indicate that the main mycotoxins (AFT, FBs, DON and ZEN) in silage does not changed in samples at 3 to 4-week sampling intervals [20]. Our results agree with those results. On the other hand, Gotlieb et al. [7] in Germany found a high occurrence of DON (643-4297 ppb), ZEN (33-51 ppb), and OT (17-37 ppb) in corn silage. In our study, we have been able to detect the co-occurrence of mycotoxins associated with adverse effects in animal. Further studies are needed to evaluate the synergism between different mycotoxins, feed safety, and quality nutrition effects on beef and cattle health.

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