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Reduction in *Fusarium* Toxin Levels in Corn Silage with Low Dry Matter and Storage Time

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Under unfavorable climatic conditions, *Fusarium* spp. can contaminate corn plants in the field and produce toxins that are present at the time of ensiling. The stability of deoxynivalenol, fumonisins B1 and B2, and zearalenone in corn silage was tested over two consecutive years. Variables studied were corn dry matter (DM) and storage length and temperature. The concentration of all *Fusarium* toxins decreased upon ensiling ($P < 0.001$). Increasing the length of storage and ensiling with low DM resulted in a higher rate of toxin disappearance, particularly for the water soluble toxins deoxynivalenol and fumonisin B1. Toxin disappearance ranged from 50% for zearalenone to 100% for deoxynivalenol. In contrast, temperature did not have any effect on stability ($P > 0.05$). These results indicate that low DM at ensiling as well as a prolonged storage could be a practical way to reduce or eliminate some *Fusarium* toxins in contaminated silages.

KEYWORDS: Whole plant corn silage; *Fusarium* toxins; stability

INTRODUCTION

Whole plant corn silage is a popular feed source for ruminants. In France, it can constitute up to 60% of the ration's dry matter (DM) in both beef and dairy cattle. Fungal contamination is the most common cause of silage losses. Whereas the genera *Aspergillus* and *Penicillium* cause problems predominantly during storage, *Fusarium* species are known to affect crops in the field throughout the world (*1–4*). Economic losses that result from *Fusarium* contamination include poor grain quality, lower crop yields, and reduced animal performance (*5, 6*). Under unfavorable climate conditions, various *Fusarium* toxins could be produced in the field and then be present in silage (*7–10*). These secondary fungal metabolites have been shown to cause a variety of toxic responses in animals, including neurological, estrogenic, hepatotoxic, and immunotoxic effects (*6, 11*). Fumonisins are also the causal agent of two well-described diseases in domestic animals, equine leukoencephalomacia (*12, 13*) and porcine pulmonary syndrome (*14*).

Although the process of fermentation reportedly can decrease the concentration of mycotoxins (*15*), there is scarce information on the stability of mycotoxins in silage. Moreover, data available are often contradictory; while some studies showed that concentration of some mycotoxins decreased during the ensiling period (*16, 17*), whereas others did not (*18, 19*). The objective of this work was to study the stability of four major *Fusarium* toxins, deoxynivalenol, fumonisin B1 and B2, and zearalenone, in corn silage stored under different conditions. The effect of dry matter content and the effect of temperature and length of storage were tested.

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MATERIALS AND METHODS

Preparation of Corn Contaminated with *Fusarium* Toxins. Chemical structures of the *Fusarium* toxins investigated in the present paper are shown in **Figure 1**. Zearalenone-contaminated corn was artificially produced using a high-zearalenone-producing strain of *Fusarium culmorum*. Briefly, corn adjusted to 50% moisture (w/w) was placed into 2 L capacity gas-permeable polypropylene bags (SACO2, Merelbeke, Belgium), autoclaved (120 °C, 20 min), and inoculated with pieces of potato−dextrose−agar cultures. Bags were sealed and incubated for 4 weeks at 28 °C. After incubation, the culture was dehydrated in a ventilated oven (45 °C, 48 h). Corn naturally contaminated with deoxynivalenol or fumonisins was obtained locally from an experimental field (Limagrain, Clermont-Ferrand, France). Corn samples were ground to pass a 1 mm screen and mixed with uncontaminated ground corn to obtain concentrations of 30 μg/g for deoxynivalenol-contaminated sample, 22 and 9 μg/g for fumonisin B1- and B2-contaminated samples, respectively, and 30 μg/g for zearalenone-contaminated sample. These concentrations were considered to

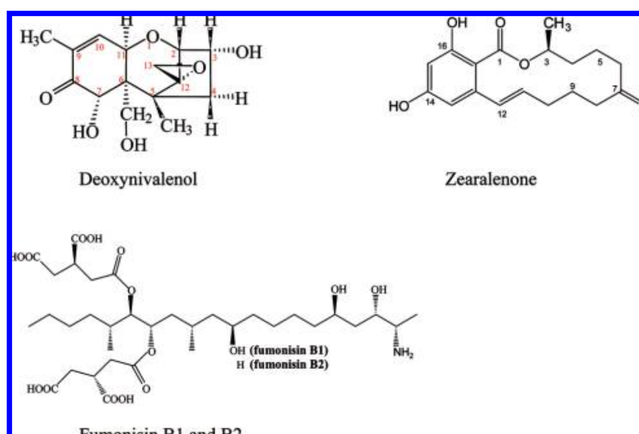

Figure 1. Chemical structures of the *Fusarium* toxins investigated.

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be the initial concentrations. The homogeneity of the contaminated corn was tested by analyzing five replicates of each ground sample. Coefficients of variation between replicates were <10%.

Ensiling Experiments. *Mini Silos.* A trial to test the most appropriate mini silo type was performed a year before the stability studies. The ensiling experiments were carried out in glass 1 L capacity (MiSi-1L) and plastic 20-L capacity (MiSi-20L) mini silos, which were compared in terms of quality of conservation and aerobic stability to large 2000 L capacity plastic bag (Si-2000L) silos, normally used in studies of homologation of silage additives. MiSi-1L silos are hermetically sealed; no air can enter, but fermentation gases can escape. On the other hand, produced juice is not eliminated. MiSi-20L silos are also hermetically closed. Like MiSi-1L, gases can escape, but effluents are collected at the bottom of the silos and separated from the plant material. The Si-2000L silos are not hermetically sealed as the MiSi, and the effluent is eliminated by making a small cut in the plastic at the bottom. Whole plant corn (cultivar ‘Anjou 265’) was harvested at 28 and 36% DM and chopped to a length of about 48−63 mm. All three different silos were packed in triplicate the same day for each DM. No additives were used at ensiling in this study. In total, 42 silos were made: 6 Si-2000L and 18 of each MiSi.

Stability Studies. The stability of *Fusarium* toxins was tested over two consecutive years on the same variety of corn, cultivar ‘Anjou 265’, ensiled at two DM levels with about 10 points of difference between the two harvests. In addition to the effect of DM, the experimental procedure tested the effect of temperature and length of storage on the toxins’ stability.

(a) Year 1. Whole plant corn was harvested at 24 and 37% DM, ensiled in Si-2000L silos ($n = 4$, two per DM) and in the selected MiSi model (MiSi-20L, $n = 18$), and stored for 11 months at three temperatures (ambient, 15 and 30 °C, 3 MiSi per temperature), but only at ambient temperature for Si-2000L. The *Fusarium* toxin-contaminated corn samples (4−5 g, $n = 180$) were placed individually into 10 × 5 cm, 200 μm porosity nylon bags (VWR, Fontenay-sous-Bois, France), spread in a fine layer, and introduced at different depths in the silos: 60, 100, and 140 cm for Si-2000L and 15 and 40 cm for MiSi-20L. Control bags (one per toxin and per silo), placed in plastic bags hermetically closed to avoid contact with silage, were also introduced into silos.

(b) Year 2. In year 2, only MiSi-20L was used ($n = 8$). Whole plant corn harvested at 28 and 42% DM was ensiled and stored at two temperatures (15 and 30 °C) during 3 and 6 months. *Fusarium* toxin-contaminated bags prepared as described above were introduced in duplicate at 15 and 40 cm depths from the bottom of the MiSi-20L. In total, 96 sample bags and 24 control bags (one per toxin and per silo) were analyzed.

Chemical Analyses. Fresh whole plant corn and silage samples were taken and analyzed for DM content and total ash. The DM content was determined in triplicate on 200 g samples spread in a metallic tray and placed in a ventilated oven at 104 °C for 24 h.

Determination of Silage Quality. The quality of silages was judged on the basis of their fermentation characteristics, DM losses, and aerobic stability. Fermentation parameters were determined from filtrates obtained after an overnight maceration at ambient temperature (*20*). Volatile fatty acid and alcohols were determined by gas chromatography (*21*), and ammonia was determined by Conway’s method (*22*). The aerobic stability of silages was determined by placing about 20 kg of a representative silage sample in a plastic box and exposing it to air. All boxes were placed in a room maintained at a constant temperature of 24.5 ± 1.5 °C. Temperature was recorded every 15 min using thermocouples placed in the center of the forage mass. Aerobic stability was defined as the time during which the temperature of the silage did not exceed by more than 2 °C the room temperature.

***Fusarium* Toxins Analysis.** After the silos were opened, nylon bags containing toxins were withdrawn and dried for 24 h at 40 °C in a ventilated oven. The dried samples were transferred to clean tubes and stored at 4 °C until analysis. Control bags were treated in the same way.

Extraction Procedure. For zearalenone, contaminated corn (0.5 g) was extracted with 10 mL of an acetonitrile/distilled water (9:1) mixture in a horizontal shaker for 45 min at 40 rpm and centrifuged at 2500*g* for 10 min. An aliquot of the extract (0.2 mL) was evaporated at 45 °C under a stream of N_2. The dried residue was redissolved in 0.2 mL of mobile phase (water/methanol, 40:60 v/v) by incubation in an ultrasonic bath for 3 min and 20 μL of this solution was injected into the HPLC system.

For deoxynivalenol, contaminated (0.5 g) corn was extracted with 10 mL of an acetonitrile/distilled water (84:16) mixture in a horizontal shaker for 45 min at 40 rpm and centrifuged at 2500*g* for 10 min. Three milliliters of the extract was applied on the top of a Multisep 225 Trich mini column (Romer Laboratories Diagnostic GmbH, Tulln, Austria), and 1 mL of the eluate was evaporated at 45 °C under a stream of N_2. The dried residue was redissolved in 0.2 mL of distilled water/acetonitrile (84:16) by incubation in an ultrasonic bath for 3 min, and 20 μL of this solution was injected into the HPLC system.

For fumonisins, contaminated corn (1 g) was extracted with 10 mL of an acetonitrile/methanol/distilled water (2.5:2.5:5) mixture in a horizontal shaker for 20 min at 40 rpm and then centrifuged at 2500*g* for 10 min. Five milliliters of the extract (corresponding to 0.5 g of the sample) was collected and subsequently added to 10 mL of phosphate-buffered saline (PBS, pH 7.4) (Sigma, Saint Quentin Fallavier, France) and centrifuged before loading onto a Fumoniprep immunoaffinity column (R-Biopharm, St Didier au Mont d’Or, France). The column was then washed with 10 mL of PBS solution, and fumonisins were eluted with 2 mL of methanol. The eluate was evaporated at 45 °C under a stream of N_2, and the dried residue was redissolved in 0.2 mL of distilled water/acetonitrile (1:1) by incubation in an ultrasonic bath for 3 min. Fumonisins were derivatized before injection using *o*-phthaldialdehyde plus mercaptoethanol. Ten microliters of extract was mixed with 90 μL of 0.1 M borate buffer at pH 10, and then 100 μL of derivatizing reagent was added. The solution was allowed to react for 2 min, and 20 μL of this solution was injected into the HPLC system.

HPLC Analysis. Separation and quantification of *Fusarium* toxins were performed at room temperature by HPLC, using UV detection for deoxynivalenol and zearalenone and fluorometry for fumonisins. The HPLC system consisted of a P1000XR pump (SpectraSYSTEM, San Jose, CA) and an automatic sampler (SpectraPhysics, San Jose, CA). Separation was performed on C_{18} reversed-phase columns: 125 × 4.6 mm, 3 μm, for zearalenone; 125 × 4.6 mm, 5 μm, for deoxynivalenol; and 150 × 4.6 mm, 3 μm, for fumonosins (Macherey-Nagel, Hoerdt, France). The mobile phase used to separate deoxynivalenol consisted of a water/acetonitrile solution (95:5), and the flow rate was 0.9 mL/min. Detection was set at 220 nm, and retention time was 6.2 min. The mobile phase used to separate zearalenone consisted of a water/methanol solution (40:60 v/v), and the flow rate was 1.2 mL/min. The detection was set at 280 nm. The retention time of zearalenone was 14.2 min. For fumonisins B1 and B2, a gradient system was used with a mixture of distilled water/methanol (1:1) at pH 3.35, and acetonitrile, at a flow rate of 1 mL/min. The gradient was started at 10% of solvent A, which increased to 60% in 6 min; this was maintained at 60% for 7 min and then returned to the initial condition in 1 min. The detection was set at 336 nm excitation and 440 nm emission. The retention times were 9.2 and 11.1 min for fumonisins B1 and B2, respectively.

Analytical Validation. The analytical methods used for this study were validated in terms of precision and accuracy. This validation was performed by analyzing replicate sets of quality control samples and certified reference materials (BIPEA, Gennevilliers, France) of known concentrations. Quality control extracts at 5 and 20 μg/g of deoxynivalenol were prepared as follows: *Fusarium* toxin-free corn was extracted with the same solvent mixture (see above), and the extracts were spiked using a specific standard solution. These criteria were also tested by using certified reference material samples. The calibration curve was determined daily, using a series of standard solutions in a mobile phase containing different levels of each toxin. The concentration of each *Fusarium* toxin was calculated by using the formula

$$\textit{Fusarium}\ \text{toxins}\ (\mu\text{g/g}) = \frac{M_g}{M_s} \quad (1)$$

where M_g is the mass of *Fusarium* toxins (μg) and M_s is the mass of the analyzed feed (g) injected into the HPLC system.

Statistical Analyses. Data were statistically analyzed by one-way analysis of variance using the General Linear Model procedure of SAS

Table 1. Main Fermentation Parameters of Whole Plant Corn Silages after 11 Months of Storage[a]

silo[b]	pH	g/kg of DM		
		ammonia	acetate	ethanol
24% DM				
Si-2000L	3.8	0.86 a	19.4 a	50.8 a
MiSi-1L	3.9	1.00 a	9.6 b	4.5 b
MiSi-20L	3.6	0.88 a	14.5 ab	38.8 a
37% DM				
Si-2000L	3.8	0.64 b	10.1 b	7.6 b
MiSi-1L	3.9	0.60 b	9.7 b	4.3 b
MiSi-20L	3.8	0.66 b	8.0 b	4.7 b
SEM	0.2	0.09	3.4	3.1

[a] Within a column, means ($n = 3$) followed by different letters differ ($P < 0.05$). SEM, standard error of the means. [b] Dry matter (DM) contents at ensiling of 1 (MiSi-1L), 20 (MiSi-20L), and 2000 L (Si-2000L) model silos.

(SAS Institute Inc., Cary, NC). The model for year 1 included silage type, dry matter, and depth of samples in the silo. For year 2 the model included dry matter, temperature, storage length, and depth of sample in the silo. The effect of sample depth was not significant, and this variable was excluded from the final analysis. Significance was declared at the 5% probability level, and differences among means were tested using the Tukey option (Tukey−Kramer method).

RESULTS

Validation of Mini Silos. Fermentation characteristics of different silages (**Table 1**) were within normal ranges (*23*) with low pH and low concentration of ammonia indicating a correct fermentation. Ethanol and acetate production were higher ($P < 0.05$) in silages at low DM. Aerobic stability for all types of silages and silos was considered good because it was higher than 40 h. However, aerobic stability in low DM was better than in high DM silages ($P < 0.05$) (data not shown). The fermentation parameters of MiSi-20L silos were similar to those of Si-2000L silos. In contrast, acetate and ethanol concentrations were lower in MiSi-1L, particularly in low DM silages ($P < 0.05$). Moreover, the hygienic quality of MiSi-20L silages was optimal, with no visible fungal development (0/18), whereas 28% (5/18) of MiSi-1L silages were completely moldy. On the basis of these results, the MiSi-20L was retained for further tests of *Fusarium* toxin stability.

Stability of *Fusarium* Toxins in Silages. The analytical methods used in this study were validated with quality control and certified reference material samples. The recovery, precision, and accuracy were considered to be satisfactory for all toxins tested (detailed information is available as Supporting Information). Chromatograms obtained after purification (fumonisins and deoxynivalenol) or without purification (zearalenone) did not show any interfering coeluting peaks with toxins (**Figure 2**). For deoxynivalenol, however, small interferences were noted in a few cases.

The concentration of all four *Fusarium* toxins tested decreased upon ensiling ($P < 0.001$). In contrast, controls placed inside the silos but avoiding contact with silage did not have any reduction in toxin concentration ($P > 0.05$). We did not observe differences in stability in samples placed at different depths in the silos, although, at low DM, samples of deoxynivalenol and fumonisin B1 placed at the lower part of the silos tended to have lower concentrations ($P < 0.15$). No important differences in stability were observed in the selected MiSi model as compared to the reference silo (**Table 2**). However, stability differed among toxins, and it was affected by silage DM and storage time. Silages with low DM content had a higher rate of toxin disappearance than silages with high DM content, particularly for the water soluble toxins deoxynivalenol and fumonisin B1 (**Table 3**). For these two toxins there was also an increased disappearance with longer lengths of storage ($P < 0.001$). Deoxynivalenol virtually disappeared after only 3 months of storage in low DM silages, whereas in high DM silages it was still present, albeit at low level, after 6 months of storage. Similar results were found with fumonisin B1, for which about 70 and 10% were recovered at 3 months of storage in silages at high and low levels of DM, respectively. In contrast, fumonisin B2 and zearalenone were more stable and not affected by storage. In high DM silages, both toxins were little affected and were recovered at approximately 80% of their initial concentration. However, at low DM the loss of fumonisin B2 accounted for about two-thirds and that of zearalenone for about half of their initial concentrations. On the other hand, storage temperature did not have any effect on the stability of *Fusarium* toxins, except for fumonisin B2, which had a lower concentration at 30 °C ($P < 0.05$). No biotransformation products were detected in chromatograms. There were no peaks corresponding to de-epoxy deoxynivalenol, aminopentol, and zearalenols—the

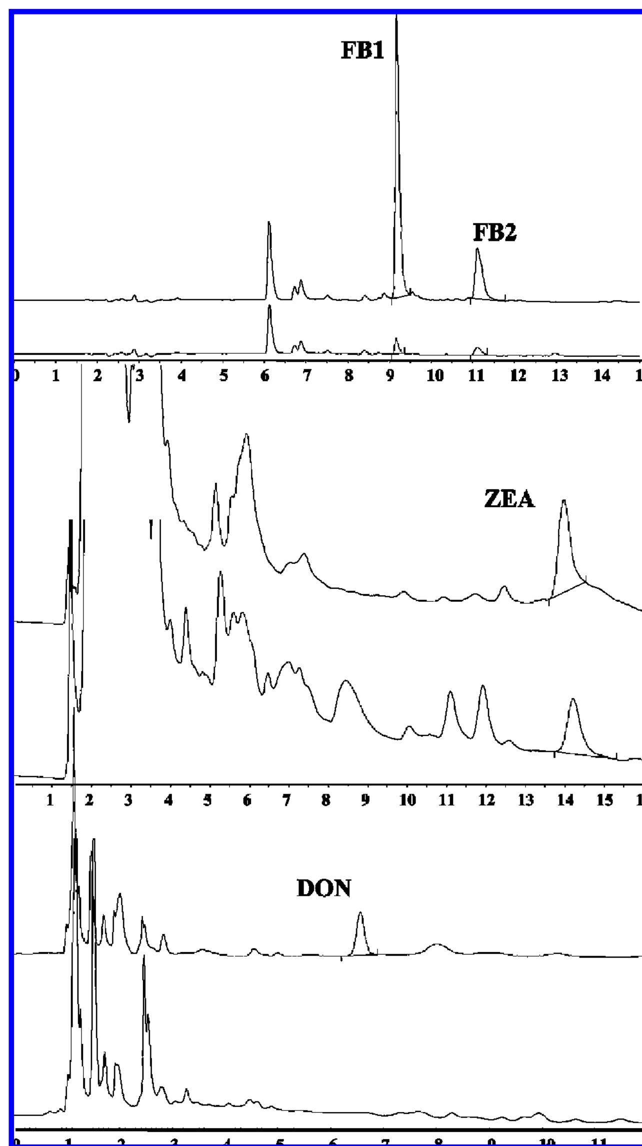

Figure 2. Typical HPLC chromatograms of an extract from *Fusarium* toxin contaminated corn after 6 months of storage in low dry matter corn silage: fumonisins B1 and B2, zearalenone, and deoxynivalenol from control (**A**) and test (**B**) samples.

Table 2. Stability of *Fusarium* Toxins in Whole Plant Corn Silages Stored at Ambient Temperature for 11 Months (Year 1)

		Fusarium toxin remaining/initial[a] (%)			
silo[b]	DM[c] (%)	deoxynivalenol	fumonisin B1	fumonisin B2	zearalenone
Si-20L	28	0.0 ± 0.0	9.7 ± 2.3	35.4 ± 2.7	60.9 ± 11.0
	38	0.0 ± 0.0	11.4 ± 1.5	27.3 ± 0.3	51.6 ± 7.6
Si-2000L	28	0.0 ± 0.0	9.1 ± 1.2	21.4 ± 9.5	65.6 ± 6.6
	38	0.0 ± 0.0	12.0 ± 6.5	34.4 ± 25.4	50.9 ± 3.6
effect of silo type		NS	NS	NS	$P < 0.05$
effect of dry matter		NS	NS	NS	NS

[a] Contaminated plant material was placed in permeable nylon bags inside the silos at initial concentrations of 30, 22, 9, and 30 μg/g for deoxynivalenol, fumonisins B1 and B2, and zearalenone, respectively. Mean ± SD, $n = 6$. [b] 20 and 2000 L silos. [c] Dry matter at ensiling time.

Table 3. Stability of *Fusarium* Toxins in Whole Plant Corn in 20 L Model Silos (Year 2)

			Fusarium toxin remaining/initial[a] (%)			
dry matter[b] (%)	storage (months)	temperature (°C)	deoxynivalenol	fumonisin B1	fumonisin B2	zearalenone
28	3	30	4.0 ± 1.7	11.1 ± 3.0	37.8 ± 6.5	60.0 ± 20.4
		15	2.4 ± 0.6	16.3 ± 7.7	54.7 ± 14.6	56.7 ± 16.6
	6	30	0.0 ± 0.0	8.3 ± 3.3	18.5 ± 2.4	56.7 ± 4.1
		15	0.0 ± 0.0	11.5 ± 2.4	73.8 ± 7.0	47.2 ± 2.9
42	3	30	16.6 ± 3.0	88.7 ± 4.3	76.7 ± 11.2	73.8 ± 7.5
		15	15.1 ± 1.3	72.2 ± 1.6	90.3 ± 3.8	79.0 ± 7.8
	6	30	5.2 ± 0.0	54.4 ± 12.0	80.6 ± 11.5	78.1 ± 0.3
		15	7.4 ± 4.5	62.2 ± 15.7	92.0 ± 6.9	94.5 ± 1.9
effect of dry matter			<0.001	<0.001	<0.001	<0.001
effect of storage length			<0.001	<0.001	NS	NS
effect of temperature			NS	NS	<0.01	NS

[a] Contaminated plant material was placed in permeable nylon bags inside the silos at initial concentrations of 30, 22, 9, and 30 μg/g for deoxynivalenol, fumonisins B1 and B2, and zearalenone, respectively. Mean ± SD, $n = 4$. [b] Dry matter at ensiling time.

main metabolites of deoxynivalenol, fumonisins and zearalenone, respectively.

DISCUSSION

All silage samples were of good quality, with low pH (<4) and low ammonia concentration. The relatively high aerobic stability of all silages can be related to the high level of acetic acid content, which improves silage preservation (*24, 25*).

The results of this study clearly indicate that the level of DM at ensiling as well as the length of storage has a major effect on the stability of *Fusarium* toxins. Similar results were obtained with alternariol and tenuazonic acid in ensiled sunflower seeds (*26*) and ochratoxin A in barley silages (*17*). The reduction in mycotoxins during the process of fermentation had been attributed to physical, chemical, and/or microbiological factors. In our study, we have observed that *Fusarium* toxin disappearance increased in low DM silages ($P < 0.001$). In contrast, temperature did not have any effect ($P > 0.05$). The stability, zearalenone > fumonisin B2 > fumonisin B1 > deoxynivalenol, followed a pattern that was inversely proportional to the molecules' physical solubility characteristics. The last two *Fusarium* toxins are more water soluble than the two former. The calculation of the "inorganic−organic balance" value (*27*) of each *Fusarium* toxin, which illustrates the lipophilic characteristic of the compound, gives the same profile: zearalenone (=1.01) > fumonisin B2 (=1.20) > fumonisin B1 (=1.65) > deoxynivalenol (=1.71). Water soluble *Fusarium* toxins might be eluted by the fermentation effluent because of their hydrophilic character. Rotter et al. (*17*) reported that the greatest absolute reduction in the concentration of ochratoxin A occurred between 0 and 7 days of ensiling. This period (1−2 weeks) corresponds to the maximum activity of lactic acid bacteria and to the production of the fermentation effluent (*28, 29*). Lauren et al. (*30*) studied the fractionation of *Fusarium* toxins in contaminated corn after wet-milling. The water soluble toxins deoxynivalenol and nivalenol were found predominantly in the liquid fraction, and the zearalenone, which is relatively insoluble, was found in the solid fraction. Other authors have also observed an appreciable reduction of fumonisin B1 content in corn after limewater processing (*31, 32*).

Additional attention should be focused on the possible biochemical conversion of mycotoxins during ensiling as elution does not fully explain the disappearance of mycotoxins. Indeed, the effect of DM became less important with longer lengths of storage (**Table 2**). Several studies have shown that some microorganisms are able to degrade or transform mycotoxins. Damoglou et al. (*33*) have reported that zearalenone was unstable in rye grass silage, and it was suggested that microbial activity during the silage process caused the breakdown of this mycotoxin. Recent works have shown that fermentative Lactobacilli are able to transform some mycotoxins (*34, 35*). In this study, whole plant corn at two DM levels was γ-irradiated (25 kGy/25 kg) before ensiling to test for the effect of epiphytic microbes on toxins' stability. Microbial analysis of silages obtained from irradiated whole plant corn after 6 months of ensiling showed that they were not completely sterile, even though a significant reduction of the level of microorganisms, especially fungi, was observed by comparison to the non-irradiated whole plant corn (data not shown).

The stability of mycotoxins in silage has been little studied. Some studies have shown that zearalenone, present in the crop at ensiling, survives the ensiling process (*18*). In contrast, other studies have reported a decrease of its concentration (*33*). These discrepancies may be due to DM differences at ensiling and to different ensiling conditions such as compression, use of

additives, and temperature and length of storage. In the published literature, there is limited information about these factors.

Silage production is not standardized, and the quality of the final product can be variable depending on the DM content and the procedure and tools used for its preparation. Because of climatic conditions, whole plant corn can be harvested and ensiled at various DM levels ranging from 20 to 45%. In addition, the process is under the control of the farmer, and the silage's hygienic quality depends on the degree of his/her technical ability. The experimental silos used in this work are a research tool for the identification of the factors and ensiling techniques that influence mycotoxin stability. A better understanding of these factors should lead to practical recommendations to reduce the risk of animal exposure. On the basis of this work, to decrease *Fusarium* toxin contamination in whole plant corn silages, it could be recommended to harvest the crop at a low DM (<28%) and to open the silo after a storage period of at least 3 months. The water soluble *Fusarium* toxins (deoxynivalenol and fumonisin B1), which are the most common and most toxic *Fusarium* metabolites, appear to be largely eliminated after a relatively short storage time of the silage. Further studies are needed to elucidate whether these toxins are actually degraded in the silage and the proportion that can be eventually eliminated in the fermentation effluent.

ABBREVIATIONS USED

DM, dry matter; MiSi, mini silo; Si-2000L, plastic bag silo; N-NH3, ammonia; PBS, phosphate-buffered saline.

SAFETY

All of the *Fusarium* toxins have important toxic effects and were manipulated following appropriate precautions.

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Supporting Information Available: Table containing the detailed information of the performance criteria of methods used for the determination of *Fusarium* toxins. This material is available free of charge via the Internet at http://pubs.acs.org.

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