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Effect of Inoculants and Enzymes on the Fermentation Characteristics, *in vitro* Digestibility and Aflatoxin of Corn Silage

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Abstract: Two additives (bacterial inoculants and enzymes) were tested for their effects on fermentation characteristics, *in vitro* digestibility, mold counts and aflatoxin content of corn silages. Whole plant corn (one half milk line) was ensiled after the following treatments: untreated (CON); bacterial inoculants (B) at 10^5 CFU g^{-1} of fresh forage; Enzymes (E) at 100 U g^{-1} of fresh forage; mixture of bacterial inoculants and enzymes at 10^5 CFU g^{-1} and 100 U g^{-1} of fresh forage, respectively (B+E). All the additives influenced the lactic acid, acetic acid, ammoniacal nitrogen content and *In vitro* Dry Matter Digestibility (IVDMD) ($p<0.05$). The E and B+E influenced the Crude Protein (CP), Neutral Detergent Fiber (NDF) content, *In Vitro* Neutral Detergent Fiber Digestibility (IVNDFD) and *In Vitro* Crude Protein Digestibility (IVCPD) ($p<0.05$). Aflatoxin content and mold counts for treated silage were lower than untreated silage ($p<0.05$) throughout the air exposure stages (0, 1, 5 and 10 days). Aflatoxin content was below the detection limit (0.01 ppb) in B treated silage after 0 day exposure to air and in B+E throughout the air exposure stages. Researchers recommend the application rates of enzymes at 100 U g^{-1} of fresh forage or bacterial inoculants plus enzymes at 10^5 CFU g^{-1} and 100 U g^{-1} of fresh forage, respectively to improve fermentation quality and aerobic stability of corn silage.

Key words: Corn silage, bacterial inoculants, enzymes, aflatoxin, mold

INTRODUCTION

Silage additives are used to improve silage quality and inoculations and enzymes are the most popular silage additives. Lactic acid bacteria were traditionally developed for the purpose of rapidly producing lactic acid and lowering pH in order to improve the efficiency of the fermentation process (Filya, 2003) and animal performance as indicated by increased milk yield, weight gain and/or feed intake (Kung *et al*., 2003). The primary function of enzymes is to break down forage fiber during fermentation which renders the silages more digestible during feedout. The breakdown of complex carbohydrates in forage into soluble sugars also helps bacteria produce lactic acid which helps to lower silage pH. Mixtures of inoculations and enzymes have also been employed to improve fermentation by releasing additional WSC from the plant cell wall or storage polysaccharides (Sheperd *et al*., 1995). However, the results from these provisional studies have been inconclusive about the effectiveness of these additives. For example, Umana *et al*. (1991) found no marked improvement after using lactobacillus additives in silages whereas Bureenok *et al*. (2005) reported that inoculants increased acidity and lowered the ammonia-N content of silages. Weinberg *et al*. (1993) reported that enzymes did not affect the fermentation of silages whereas Sheperd and Kung (1996) found that enzymes improved fermentation by providing extra substrate. Therefore, production must be evaluated before these additives become widely used.

The stability of silages after exposure to air is also a very important factor in determining its subsequent nutritional quality and feeding value (Filya, 2004). All silages exposed to air will deteriorate, due to aerobic microbial activity which can negatively influence silage quality and farm profitability (Tabacco *et al*., 2009) and even produce mycotoxins such as aflatoxin. Aflatoxin is a strong chemical carcinogen with strong teratogenicity and mutagenicity and is mainly produced by *A. flavus* and *A. parasiticus* (Richard *et al*., 2009). Maing *et al*. (1973) were the first to study the effect of lactic acid bacteria on aflatoxin. Coallier-Ascah and Idziak (1985), Karunaratne *et al*. (1990) and Gourama and Bullerman (1995) reported that lactic acid bacteria could produce

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metabolins that affected the biosynthesis of aflatoxin. El-Nezami *et al.* (1998) summarized the restraint mechanisms used by lactic acid bacteria on aflatoxin and suggested that three mechanisms were involved: reducing fungal growth by lowering the pH; producing metabolites that restrict fungal growth or transform the mycotoxin into innocuous or harmless compounds; combine with the aflatoxin. However, all of these studies were based on microorganism culture methods. There have been few studies that have concentrated on silage.

One objective of this study was to determine the effect of two additives on the fermentation characteristics of corn silages. A second objective was to investigate if bacterial inoculants and enzymes could reduce aflatoxin production during silage exposure to air.

MATERIALS AND METHODS

Corn and ensiling: Corn (Keduo No. 8 from CAS, China) was grown at the Research Station of China Agriculture University, Inner Mongolia (43°56′N, 118°03′E) in April 2011. The temperature range and total precipitation during the growing season were 17.7-27.9°C and 50 mm, respectively. The corn was harvested at the one half milk line stage (in mid August) and chopped into 10 mm lengths using a conventional forage harvester. The chopped forages were mixed and divided into equal portions so that the four treatments could be applied. These were: untreated (CON); bacterial inoculants, B, *Lactobacillus rhamnosus* >6×10^{10} cfu g^{-1}, Lallemand, Canada) at 10^5 cfu g^{-1} of fresh forage; enzymes (E, cellulase, glucosidase, avicelase and xylanase, 30000 U g^{-1}, Snowbrand, Japan) at 100 U g^{-1} of fresh forage and a mixture of bacterial inoculants (*Lactobacillus rhamnosus* >6×10^{10} cfu g^{-1}, Lallemand, Canada) at 10^5 CFU g^{-1} of fresh forage and enzymes (cellulase, glucosidase, avicelase and xylanase, 30000 U g^{-1}, Snow brand, Japan) at 100 U g^{-1} of fresh forage (B+E). The additives were diluted in deionized water and applied using a hand sprayer at the rate of 2 mL kg^{-1} of corn. The same deionized water was sprayed onto the untreated corn. Each treatment was replicated four times. All treatments were ensiled in bags (1 m^3) used for ensiling (approximately 600 g m^{-3}), vacuum sealed and stored in the laboratory at an ambient temperature (25±2°C) for 60 days.

After opening the bags, the silages were divided into two parts. One part (about 100 g) was used for determining fermentation and *in vitro* digestibility. The other was subjected to mold counts and aflatoxin testing after 0, 1, 5 and 10 days exposure to air. This part (about 500 g) was loosely placed in a polystyrene box and allowed to aerobically deteriorate at a constant room temperature (25±2°C). The top and bottom of the boxes contained a 2 cm diameter hole which allowed air to enter and CO_2 to leave. The silages were sampled after 0, 1, 5 and 10 days of exposure to air in order to determine the mold counts and aflatoxin content.

Determination methods: The fermentation indices were measured using the following methods: 20 g of the sampled silages were homogenized in 180 mL of distilled water for 1 min at high speed (12000 r min^{-1}). The resulting suspension was filtered through four layers of cheesecloth, centrifuged for 20 min at 27,500×g and the pellet discarded. Samples of the supernatant were used for pH, lactate, VFA (lactic acid, acetic acid, propionic acid and butyric acid) and NH_3-N analyses. The pH of the filtrate was measured with a pH meter (PHS-3C). Lactic acid, acetic acid, propionic acid and butyric acid were measured by HPLC (SHIMADZE-10A, Shimadze, Japan) according to Owens *et al.* (1999). The HPLC System consisted of a Shimadzu System Controller (SCL-10A) and a Shodex Rspak KC-811 S-DVB gel Column (300×8 mm) at a column temperature of 50°C. The mobile phase was a solution of 3 mmol perchloric acid at a rate of 1 mL min^{-1}. The injection volume was 50 μL. A UV detector (SPD-10A) was used and analyses were carried out at 210 nm. Ammonia-N (NH_3-N) was determined by the Pbenol-Hypochlorite Colorimetric Method according to Broderick and Kang (1980) and DM was determined by oven drying at 65°C for 48 h. Crude Protein (CP) was determined using the Kjeldahl Method (AOAC, 1995) and Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) were analyzed according to Van Soest *et al.* (1991). Water-Soluble Carbohydrate (WSC) was determined by the Deriaz (1961) Method. Starch concentration was determined according to the methods of the Association of Official Analytical Chemists (AOAC, 1995) using a K-AMYL assay kit (Megazyme International, Bray, Ireland). A two-step approach was used to determine *In Vitro* Dry Matter Digestibility (IVDMD), *In Vitro* Crude Protein Digestibility (IVCPD), *In Vitro* NDF Digestibility (IVNDFD) and *In Vitro* ADF Digestibility (IVADFD) (Tilley and Terry, 1963). Mold counts were enumerated by pour plating in Standard Methods (M124) agar containing 40 mg kg^{-1} of chloramphenicol and chlortetracycline (Bandler *et al.*, 1998). Aflatoxin was determined using the Immunoaffinity column (Aflatest) method of the Association of Official Analytical Chemists (AOAC, 1995).

Statistical analysis: Microbial data were converted to $\log_{10}$ and presented on a fresh weight basis. The mold

counts and aflatoxin content after 0, 1, 5 and 10 days exposure to air were analyzed using the GLM procedure according to the model for a 3×3 factorial treatment design:

$$Y_{ij} = \mu+I_i+T_j+(I\times T)_{ij}+e_{ij}$$

Where:

Y_{ij} = Dependent continuous variable
μ = Overall mean
I_i = Effect of additives
T_j = Effect of time of air exposure
$(I\times T)_{ij}$ = Effect of interaction between the additives and the time of air exposure
e_{ij} = Error term

The fermentation indices, chemical composition and *in vitro* digestibility at bag opening were analyzed for their statistical significance via ANOVA using the SPSS Version 16 (SPSS Inc., Chicago, IL). Differences among means of treatments were compared by Tukey range test. The differences were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

The chemical compositions (DM basis) of fresh chopped whole plant corn prior to ensiling are shown in Table 1. It had DM content of 227 g kg^{-1}, starch ADF of 558 and 255 g kg^{-1}. Additionally, it also had 558 g kg^{-1} of hemicelluloses and <0.0001 ppb of aflatoxin.

Table 1: Chemical composition of fresh whole-plant corn before ensiling

Compositions	Corn	SE
DM (g kg^{-1})	227	0.20
Starch (g kg^{-1} DM)	187	0.40
WSC (g kg^{-1} DM)	66.3	0.17
CP (g kg^{-1} DM)	113	0.10
NDF (g kg^{-1} DM)	558	1.10
ADF (g kg^{-1} DM)	255	0.50
Hemicelluloses (g kg^{-1} DM)	303	0.80
Aflatoxin (ppb of DM)	<0.0001	-

DM: Dry Matter; WSC: Water-Soluble Carbohydrate; CP: Crude Protein; NDF: Neutral Detergent Fiber and ADF: Acid Detergent Fiber. Hemicellulose: NDF-ADF

The fermentation quality and chemical compositions of the corn silages after 60 days of conservation are of 187 shown in Table 2. The pH value of all silages at bag opening was below 4.0 and E and B+E were lower than CON ($p<0.05$). The treated silages had higher ($p<0.05$) lactic acid and acetic acid contents than the CON whereas the ratio of lactic/acetic acid in B treatment was higher compared to the CON ($p<0.05$). Traces of propionic acid were found in this study but butyric acid was below the detection limit (<0.01% of DM) in all silages. All treatments influenced the ammoniacal nitrogen (NH_3-N) content ($p<0.05$). The E and B+E influenced DM, CP and NDF contents ($p<0.05$) but E also increased WSC content ($p<0.05$). However, there were no significant differences in starch and ADF contents between treated and untreated silages ($p<0.05$).

In vitro digestibilities of corn silages are reported in Table 3. All the additives treated silages increased IVDMD ($p<0.05$) compared with the CON. The E and B+E also significantly increased ($p<0.05$) IVCPD and IVNDFD. However, none of the additives influenced IVADFD.

The aflatoxin content and mold counts of the silages after exposure them to air are shown in Table 4. The mold counts in the treated silages were lower ($p<0.05$) than the CON throughout the period of air exposure. After 0 day exposure, all the additives especially B and B+E had lower aflatoxin contents than the CON ($p<0.05$). Lower aflatoxin levels were also detected in the treated silages compared with the CON after 1, 5 and 10 days exposure ($p<0.05$). The aflatoxin contents of the B+E silages were below the detection limit (0.01 ppb) throughout the air exposure stages.

The DM contents of whole-plant corn in this study were below the minimum DM content of 24.7% recommended by Castle and Watson (1973) to ensure successful ensiling. In order to improve fermentation, high density ensiling was used (approximately 600 g^{-3}) in this study. WSCs were within the range needed to obtain

Table 2: Fermentation quality of whole-plant corn silages treated with or without additives

	Treatments					
Compositions	CON	B	E	B+E	SEM	p-value
pH	3.78^{a}	3.8^{a}	3.66^{b}	3.65^{b}	0.031	0.005
Lactic acid (g kg^{-1} DM)	71.70^{c}	90.5^{b}	95.00^{b}	101.60^{a}	0.240	<0.001
Acetic acid (g kg^{-1} DM)	21.70^{c}	23.9^{b}	26.60^{b}	29.00^{a}	0.150	0.007
Lactic:Acetic acid Ratio	3.30^{b}	3.8^{a}	3.60^{ab}	3.50^{ab}	0.170	0.021
Propionic acid (g kg^{-1} DM)	0.90	0.9	0.70	0.80	0.010	0.133
NH_3-N (g kg^{-1} TN)	16.80^{a}	14.3^{b}	13.60^{b}	13.20^{b}	0.100	0.024
DM (g kg^{-1})	250.00^{a}	243.0^{a}	222.00^{b}	218.00^{b}	0.600	0.002
WSC (g kg^{-1} DM)	9.70^{b}	9.9^{b}	12.50^{a}	10.70^{b}	0.080	0.005
Starch (g kg^{-1} DM)	196.00	203.0	200.00	202.00	0.400	0.245
CP (g kg^{-1} DM)	114.00^{b}	118.0^{b}	121.00^{a}	119.00^{a}	0.400	0.082
NDF (g kg^{-1} DM)	527.00^{a}	510.0^{a}	497.00^{b}	504.00^{b}	0.700	0.016
ADF (g kg^{-1} DM)	267.00	273.0	262.00	253.00	0.800	0.131

Within each row, means followed by different letters differ significantly ($p<0.05$); CON: Control, no additive; B: Bacterial inoculant; E: Enzyme; B+E: Bacterial inoculants plus enzyme

Table 3: *In vitro* digestibility of whole-plant corn silages treated with or without additives

	Treatments					
Compositions (g kg^{-1})	CON[1]	B	E	B+E	SEM	p-value
IVDMD	506.9^{c}	533.5^{b}	578.0^{a}	567.2^{a}	0.80	<0.001
IVCPD)	492.5^{b}	501.1^{b}	566.9^{a}	586.4^{a}	0.58	<0.001
IVNDFD	414.5^{b}	424.2^{b}	484.1^{a}	471.1^{a}	0.67	<0.001
IVADFD	273.5	273.4	282.5	269.8	0.71	0.160

Within each row, means followed by different letters differ significantly ($p<0.05$); CON: Control, no additive; B: Bacterial inoculant; E: Enzyme inoculant B+E: Bacterial inoculants plus enzyme

Table 4: Changes in aflatoxin contents and mold counts for the silages treated with or without additives after air exposure

		Treatments					
Contents	Air exposure (day)	CON	B	E	B+E	SEM	p-value
Molds (lg cfu g^{-1})	0	1.89^{a}	1.05^{c}	1.46^{b}	0.55^{e}	0.07	<0.001
	1	2.24^{a}	1.76^{c}	2.05^{b}	0.84^{d}	0.12	<0.001
	5	3.78^{a}	2.12^{c}	2.63^{b}	1.02^{d}	0.04	<0.001
	10	4.69^{a}	3.41^{b}	3.55^{b}	1.51^{c}	0.10	<0.001
Aflatoxin content (ppb of DM)	0	0.32^{a}	0.00^{c}	0.13^{b}	0.00^{c}	0.02	<0.001
	1	0.64^{a}	0.15^{c}	0.33^{b}	0.00^{d}	0.02	<0.001
	5	2.47^{a}	1.08^{c}	1.69^{b}	0.00^{d}	0.02	<0.001
	10	6.92^{a}	2.98^{b}	3.04^{b}	0.00^{c}	0.04	<0.001

Within each row, means followed by different letters differ significantly ($p<0.05$); In the GLM procedure, the effects of additives, day and additives x day were highly significant for all parameters; CON: Control, no additive; B: Bacterial inoculant; E: Enzyme inoculant; B+E: Bacterial inoculants plus enzyme

good fermentation rates (Rooke and Hatfield, 2003) and the aflatoxin content was under the detection limit (0.01 ppb).

Successful ensiling requires epiphytic Lactic Acid Bacteria (LAB) and WSC to produce sufficient lactic acid for rapid pH reduction (McDonald *et al*., 1991). The primary fermentation acids were lactic acid and acetic acid in this study, the pH value of all the silages was below 4.0 and there was no butyric acid which indicated that the growth of undesirable microorganisms had been successfully restricted at the beginning of ensiling (Arriola *et al*., 2011). The ratio of lactic acid and acetic acid in B was higher than in the CON, reflecting its higher capacity for lactic acid production. The NH_3-N/TN of treated silages in this study was lower than the CON. This is consistent with earlier results of Stokes (1992) and Cai *et al*. (1997) who both reported that microorganisms or enzyme additives could reduce NH_3-N/TN content. Ammoniacal Nitrogen (NH_3-N) is produced by rot bacteria and reflects the degradation levels of proteins and amino acids during ensiling. The low NH_3-N concentration may be attributable to the sharp decline in pH which would rapidly inhibit aerobic microorganisms and plant enzymes, resulting in a reduction in protein degradation during the fermentation process.

The WSC concentration in the silages in this study were lower than in the fresh crops, probable because WSC was consumed by plant metabolism and microbial activity during ensiling. The purpose of adding cell wall degrading enzymes during ensiling such as cellulases and hemicelluloses is to change the cell wall content into WSC for lactic acid bacteria utilization. Ozduven *et al*. (2009) also reported that a mixture of lactic acid bacteria and enzymes decreased neutral detergent fiber content. Thus, higher concentrations of WSC were seen in the E treated silages in this study and lower NDF concentrations in E and B+E treated silages, compared with the control. The NDF and ADF results in the B treated silages in this study contradicted the results of earlier research (Baytok *et al*., 2005; Filya, 2003) which did not observe a reduction in the cell-wall fractions for inoculated silages, compared to the control. The reason may be that these substrates cannot be used by lactic acid bacteria directly. The increased CP content in E and B+E in this study could be attributed to higher DM losses in these silages, compared to the control.

Bacterial inoculation in this study improved the *in vitro* digestibility of DM. This is consistent with the study of other researchers (Harrison *et al*., 1989; Aksu *et al*., 2004) who reported increased DM digestibility in grass, alfalfa and corn silages after inoculation but contradicts other studies that reported a lack of improvement in the digestibility of silage after inoculation (Sanderson, 1993; Filya, 2003). However, Tengerdy *et al*. (1991) reported that the IVNDFD of alfalfa silages could be improved by enzymes and Sheperd and Kung (1996) showed that the treatment of maize (*Zea mays* L.) silage with an enzyme additive improved *in vitro* NDF digestion. This study's results were consistent with some of the earlier studies. Ozduven *et al*. (2010) also reported that enzymes and a lactic acid bacteria plus enzymes mixture decreased neutral detergent

fiber content and increased the *in vitro* dry and organic matter digestibility of silages which was consistent with the results of this study.

Aerobic deterioration of silage is a complex process which depends on many factors. During exposure to air, fermentation acids and other substrates are oxidized by aerobic bacteria, yeasts and molds (McDonald *et al*., 1991). The treatments affected mold counts and aflatoxin content in this study. Mold counts in treated silages were lower than the CON from 0-10 days exposure. Kleinschmit *et al*. (2005) found that the reduced fungal populations in silages treated with LAB or LAB-enzymatic additives. This suggests that the additives influenced the growth and reproduction of molds. Alflatoxin production requires many factors such as oxygen, temperature and moisture (Bandler *et al*., 1998) to be right. Based on the result that aflatoxin content before corn ensiling was below the detection limit (0.01 ppb) it can be concluded that the aflatoxin detected after 0 day exposure was produced during ensiling and the effects of B and B+E were better than E after 0 day exposure. This indicated that the environment present in the bacterially (B and B+E) treated silages influenced aflatoxin production during ensiling. Higher aflatoxin was detected after 1, 5 and 10 days exposure compared with 0 day exposure in all silages except the B+E (<0.01 ppb). Furthermore, the aflatoxin content in treated silages was significantly lower than in untreated silages after 1, 5 and 10 days exposure. This suggests that the additives especially B+E, influenced the production of aflatoxin during exposure to air by maintaining a lower pH value in the silages which led to the production of metabolites from lactic acid bacteria that helped restrain the fungus, transform the mycotoxin into innocuous or harmless compounds or combined it with lactic acid bacteria (El-Nezami *et al*., 1998).

CONCLUSION

The application of enzymes or bacterial inoculants plus enzymes accelerated the fermentation process, improved the IVDMD, IVCPD and reduced the aflatoxin contents and mold counts for whole plant corn silages. In addition, E and B+E reduced the NDF of the silages and increased the IVNDFD. The recommended ratio of enzymes is 100 U g^{-1} of fresh forage 4-8% of FW while bacterial inoculants plus enzymes at 10^5 CFU g^{-1} and 100 U g^{-1} of fresh forage, respectively produce high-quality with a good aerobic stability.

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