Journal of Animal and Feed Sciences, 25, 2016, 174–178
The Kielanowski Institute of Animal Physiology and Nutrition PAS, Jabłonna

Short communication

Effect of hybrid, inoculant and storage time on whole plant nitrogen compounds and grain total zein content in maize silage

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KEY WORDS: maize silage, nitrogen compounds, grain, zein content, storage time, inoculant

Received: 19 October 2015
Revised: 7 March 2016
Accepted: 20 May 2016

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ABSTRACT. The study examined the influence of hybrid, long-term storage and inoculants addition on fermentation profile and grain total zein content in whole plant maize (*Zea mays* L.) ensiled in laboratory silos. Three yellow maize hybrids were grown under identical conditions in a split-plot field test in five replicates. Replicates were either untreated or mixed with the lactic acid bacterial additive (Sil-All 4x4, Danstar Ferment, Zug, Switzerland) at a concentration of 1 x 10^6 CFU · g^{-1}, then ensiled in 30-dm^3 laboratory silos for one year. Silages were sampled after 3, 8 and 52 weeks, when whole plant crude protein, soluble crude protein, ammonium-N, lactic acid and grain total zein contents were assayed. Although crude protein level did not vary significantly during one-year storage, soluble crude protein and ammonium-N contents increased, while grain total zein content decreased. Addition of inoculant was associated with higher lactic acid concentration and lower grain total zein content at all time points. The greatest grain total zein degradation was found after week 52, and this growth was accompanied by a decline in lactic acid content. Trends were similar across the three tested hybrids, though the magnitude of changes differed significantly. The simultaneous decrease in grain total zein content and lactic acid content strongly suggests proteolytic zein degradation during prolonged storage.

Introduction

Nitrogen compounds are important indicators of silage quality (Der Bedrosian et al., 2012; Ferraretto et al., 2015). In particular, whole plant maize silage so-luble crude protein (sCP) and ammonium-N (NH_3-N) contents are positively associated with proteolysis (Winters et al., 2000; Johnson et al., 2002; Slottner and Bertilsson, 2006) and may be negatively associated with zein content (Hoffman et al., 2011). Zein proteins surround starch granules in maize grain and their hydrophobic nature acts as a barrier to starch digestion by rumen microbes (Giuberti et al., 2014). Below et al. (2004) reported that total zein content of maize grain depends primarily on genotypic factors; with environmental factors such as N supply or plant maturity at harvest both causing smaller or larger phenotypic variations.

Changes in nitrogen compounds during ensiling of whole plant or high-moisture maize have been examined to some extent (Johnson et al., 2002; Slottner and Bertilsson, 2006; Ferraretto et al., 2015), but detailed insights are lacking, especially into changes occurring during a one-year stable phase, such as how grain total zein content changes in whole plant silage. Der Bedrosian et al. (2012)

found that proteolytic mechanisms, which can significantly change silage quality, remain active even after 2 – 3 months of storage; those authors concluded that maize silages should be monitored throughout the storage period.

In silage production, lactic acid bacteria (LAB) additives are widely used to optimize fermentation and thereby maximize lactic acid production, quickly reduce pH and preserve nutrients (McDonald et al., 1991). Such optimization reduces proteolysis during ensiling (Winters et al., 2000; Slottner and Bertilsson, 2006; Fijałkowska et al., 2015). Not all inoculants work equally well: Filya (2003) found that inoculation with *Lactobacillus buchneri* alone resulted in greater ammonium-N content and fermentation loss, whereas inoculation either with *Lactobacillus plantarum* alone or with the combination of *L. plantarum* and *L. buchneri* reduced the proteolysis effectively. Much more work is needed to identify which inoculants have better effects on silage nitrogen compounds.

The present study examined the influence of inoculant addition on crude protein, ammonium-N, soluble crude protein and grain total zein contents in silages of three yellow maize hybrids at different time points during the long-term stable phase. To our knowledge, this is the first detailed report on grain total zein degradation in whole plant maize silage during the long-term stable phase.

Material and methods

Silage preparation

The three random yellow maize hybrids widely used in silage production in Croatia (hybrids A, B and C; Bc Institute d.o.o., Zagreb, Croatia) were analysed in this study. The hybrids were grown in 2013 under the same conditions in split-plot experimental fields at the Faculty of Agriculture of the University of Zagreb (Croatia). Each hybrid was grown in five replicates, with each replicate occupying 14 m^2 and fertilized with 400 kg · ha^{-1} of NPK 7-20-30 (N-P_2O_5-K_2O). Starter fertilizer was urea (100 kg · ha^{-1}), and KAN N (MgO) was applied twice during vegetation (175 kg · ha^{-1}). All three maize hybrids were harvested in the milk-wax stage of growth (one-half to three-quarters of the milk line), and dry matter (DM) content ranged from 33.20 to 35.97%. The hybrids were chopped mechanically using a GB370 chipper (VIKING, Langkampfen/Kufstein, Austria) that resulted with average mean particle size of 7.1 mm (Heinrichs and Kononoff, 2002).

Chopped material from each replicate was divided in half; one part was left untreated while the other one was treated with the LAB additive Sil-All 4 × 4 (Dansar Ferment, Zug, Switzerland) at a concentration of 1×10^6 CFU · g^{-1}. This additive supplies four LAB (*Lactobacillus plantarum* CNCN I-3235, *Enterococcus faecium* CNCN I-3236, *Pediococcus acidilactici* CNCN I-3237, *Lactobacillus salivarius* CNCN I-3238) and four enzymes [*Bacillus subtilis* α-amylase (EC 3.2.1.1), *Trichoderma longibrachiatum* cellulose (EC 3.2.1.4), *Aspergillus niger* hemicellulose (EC 3.2.1.8) and *Aspergillus niger* xylanase (EC 3.2.1.8)]. These enzymes are used to release naturally occurring sugars in order to speed up the fermentation. The additive (0.1 g) was diluted in 0.02 dm^3 of distilled water and sprayed over 10 kg of chopped material. The silages were packed manually into 30 dm^3 laboratory-scale silos equipped with gas blowout. An average silage packing density was 211.58 kg DM per m^3. The silages were maintained at 25 ± 2 °C and sampled after 3, 8 and 52 weeks. Week 3 was selected as the first sampling because it corresponds to the beginning of the stable phase (Bolsen et al., 1996). The collected samples were stored at –20 °C until further chemical analysis.

Chemical analysis

Silage samples were thawed for 3 h at 4 °C, and then divided in three subsamples. One subsample was subjected to DM determination (100 g, 103 °C, 24 h) and preparation of silage water extract (Nishino and Uchida, 1999). Whole plant crude protein was determined from the same thawed subsample to avoid ammonium evaporation, using the Kjeldahl procedure to assay total N. The total N value was multiplied by 6.25 to obtain the crude protein. Lactic acid concentration was determined colorimetrically from silage water filtrate (Madrid et al., 1999). NH_3-N concentration in silage was determined by distilling 20 ml of filtrate on an automated Kjeltec™ 2200 system (FOSS, Hillerød, Denmark), then colorimetrically assaying NH_3-N in the distillate using a modified Nessler method (Merck, 1974).

The second subsample of the thawed sample was manually separated and the grain sample from whole plant silage was prepared to analyse grain total zein and grain DM content (2 g, 103 °C, 24 h). The grain sample before zein detection was dried (60 °C, 24 h), grinded to pass through the 1-mm screen of a cyclone mill (Cyclotec 1093, Foss Tecator, Hoganas, Sweden) and analysed for DM (2 g, 103 °C, 4 h). Grain total zein extraction was performed according to Wallace et al. (1990). Total N in the zein extract

was determined using the Kjeldahl procedure with modification in concentration of HCl (0.03 M instead of 0.1 M). The resulting value was multiplied by 6.25 to obtain grain zein content. The third subsample was used to prepare dried whole plant silage sample (60 °C, 24 h). Dried sample was grinded (1 mm, Cyclotec 1093, Foss Tecator, Hoganas, Sweden) and analysed for both DM (4 g, 103 °C, 4 h) and sCP. The 'NorFor' procedure of the Nordic feed evaluation system was used to measure sCP content (Åkerlind et al., 2011).

Statistical analysis

Data were analysed at the level of split-plot over time using the PROC MIXED procedure in SAS 9.3 (SAS Institute, Cary, NC, USA). Main effects in the model included maize hybrid, inoculant addition and storage time, as well as interactions among these variables. The intercepts of hybrid and inoculant application over plots were treated as a random model effect, while time was treated as a repeated statement. Means were defined using the least squares means statement, and compared using the PDIFF option. PDMIX macro in SAS was used for defining superscript letter groups (Saxton, 1998). The differences of green material among the tested hybrids were performed separately as a split-plot completely randomized design and differences between means were assessed for significance using the PDIFF option. Differences (Table 2) and interactions (Table 2; Figure 1) were considered significant when $P < 0.05$.

Results

The tested maize hybrids before ensiling showed significant differences in whole plant crude protein, grain total zein and sCP content (Table 1). Hybrids B and C were similar in sCP and grain total zein contents. Hybrid A had the highest whole plant crude protein, sCP and grain total zein content.

Table 1. Nitrogen compounds and lactic acid content in whole plant, and grain total zein content in maize hybrids before ensiling

Hybrid	Crude protein	sCP	NH_3-N, % N	Lactic acid,	Grain total zein,
	g ·kg^{-1} DM			g ·kg^{-1} DM	g ·kg^{-1} grain DM
A	79.60^{a}	18.85^{a}	3.74	3.92	76.36^{a}
B	62.34^{c}	16.70^{b}	3.60	6.84	63.53^{b}
C	70.21^{b}	16.17^{b}	3.78	3.17	65.79^{b}
P	<0.001	0.003	0.958	0.240	0.027
SD	2.96	0.34	0.50	1.53	3.07

DM – dry matter; sCP – soluble crude protein; ^{abc} – means with different superscripts within a column are significantly different ($P < 0.05$) according to Tukey's test

Table 2. Effect of storage time (T), hybrid (H) and inoculant (I) application on nitrogen compounds, lactic acid and grain total zein content in whole plant maize silage

Indices		Crude protein	sCP	NH_3-N, % N	Lactic acid,	Grain total zein, g ·kg^{-1}
		g ·kg^{-1} DM			g ·kg^{-1} DM	grain DM
Storage time	3	66.43	23.91^{c}	4.16^{c}	29.11^{b}	63.97^{a}
(T), week	8	66.22	25.85^{b}	4.65^{b}	30.28^{a}	59.93^{b}
	52	69.43	31.86^{a}	7.33^{a}	19.07^{c}	39.83^{c}
Effect of storage time, *P*		0.207	<0.001	<0.001	<0.001	<0.001
SD		1.84	0.79	0.40	1.95	1.59
Hybrid (H)	A	73.79^{a}	31.14^{a}	5.88	29.96^{a}	58.58
	B	63.21^{b}	25.81^{b}	4.83	24.26^{b}	51.31
	C	65.07^{b}	24.67^{b}	5.43	24.22^{b}	53.84
Effect of hybrid, *P*		<0.001	<0.001	0.364	0.020	0.158
SD		1.71	0.59	0.51	1.53	2.50
Inoculant (I)						
not added		68.51	27.23	5.50	21.57^{b}	55.80^{a}
added		66.21	27.14	5.27	30.73^{a}	53.35^{b}
Effect of inoculant, *P*		0.136	0.826	0.288	<0.001	0.047
SD		1.51	0.52	0.32	1.19	1.52
Interactions						
T × H, *P*		0.596	0.072	0.573	0.096	0.266
T × I, *P*		0.081	0.841	0.607	0.007	0.046
H × I, *P*		0.252	0.107	0.948	0.430	0.815
T × H × I, *P*		0.986	0.391	0.163	0.199	0.082

DM – dry matter; sCP – soluble crude protein; ^{abc} – means with different superscripts within a column are significantly different for each item ($P < 0.05$) according to Saxton (1998)

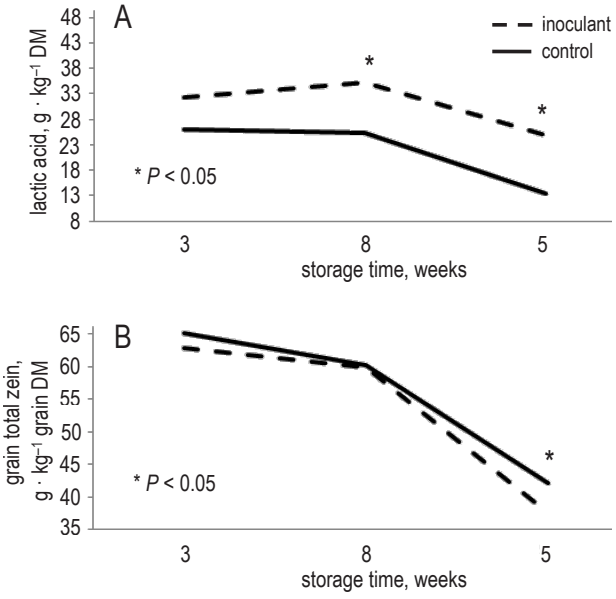

Figure 1. Interactions between inoculant application and storage time on A. lactic acid content and B. grain total zein content in whole plant maize silages during prolonged storage. Results for all three hybrids were averaged together. The asterisk (*) indicates mean values of samples with added inoculant that differ significantly ($P < 0.05$) from samples without added inoculant for each storage time separately (Saxton, 1998)

The whole plant crude protein content in silages did not change during the one-year storage ($P = 0.207$), regardless of the inoculant addition ($P = 0.136$). The highest whole plant crude protein content during storage was observed in hybrid A silages ($P < 0.001$). The sCP and NH_3-N contents significantly increased in silages during storage ($P < 0.001$; Table 2). The sCP content varied significantly between hybrids ($P < 0.001$), with hybrid A silages containing the highest sCP content. The NH_3-N content (Table 2) did not vary significantly between hybrids ($P = 0.364$). Neither sCP nor NH_3-N contents in silages differed significantly in the presence or absence of inoculant additive (Table 2).

Lactic acid content in silages (Table 2) varied significantly with storage time ($P < 0.001$) and genotype ($P < 0.05$), and was significantly higher in silages containing inoculant than in those without it ($P < 0.001$; Figure 1A). In all hybrids the lactic acid content was the highest at week 8 (Table 2), after which it decreased. Grain total zein content decreased during long-term storage ($P < 0.001$), reaching its lowest level at week 52 (Table 2; Figure 1B) regardless of maize genotype ($P = 0.158$). Zein content was similar between inoculated silages and control ones at weeks 3 and 8, but was significantly lower in inoculated silages at week 52 ($P < 0.05$).

Discussion

The obtained data show that in contrast to specific nitrogen compounds, the whole plant crude protein content did not change in any of the hybrids during the one-year storage and agrees with the findings of Ferraretto et al. (2015). Our data also suggest that when production conditions and plant maturity at ensiling are held constant, hybrid genotype is the primary determinant of whole plant crude protein content in both green material and silages. This finding is consistent with work by Johnson et al. (2002).

Lactic acid production during ensiling of forages is well established (McDonald et al., 1991); this most abundant and the strongest acid in silages dissolves zein proteins, leading to a reduction in grain total zein content (Lawton, 2002). In addition to lactic acid, enzymes from plants or microorganisms degrade zein proteins (McDonald et al., 1991; Lawton, 2002; Hoffman et al., 2011). As a result of both types of degradation, the total zein content was lower in silage grain than in the grain before ensiling. Lactic acid levels in our study decreased from week 8 to week 52, while grain total zein content reached its lowest level at week 52. The observed reduction in lactic acid content with continued drop in grain total zein content after 8 weeks suggests that proteolysis was primarily responsible for degradation of grain zeins later during storage. Our findings are consistent with the hypothesis of Hoffman et al. (2011) that zein degradation in high moisture maize silage primarily reflects proteolysis. Lactic acid content often decreases during the long-term stable phase and one possible cause is LAB utilization of lactic acid under conditions of hexose limitation (McDonald et al., 1991). The processes affecting lactic acid content under the presented ensiling conditions are recommended to be examined in the future studies.

Further evidence of proteolysis-mediated zein degradation is our observation that the sCP and NH_3-N contents increased continuously in all hybrids during the long-term stable phase. Both parameters are positively associated with proteolysis (Winters et al., 2000; Johnson et al., 2002; Slottner and Bertilsson, 2006). Such results suggest the recommendation that maize silages should be assayed before feeding throughout the whole storage period.

Our data show that adding inoculant to whole plant silage increased grain total zein degradation during the long-term stable phase, especially in the later stages. This effect presumably reflects LAB activity, since the four enzymes in the inoculant degrade only carbohydrates (McDonald et al., 1991). Zein protein degradation is expected to increase vulnerability of starch granules to enzymatic degradation (Giuberti et al., 2014) which can affect animal performance. Future studies should focus on such performance examination during long-term stable phase in the presence and absence of inoculant.

Conclusions

The present study provides evidence that adding inoculant to maize silage can increase grain total zein degradation during the long-term storage, and that declines in lactic acid concentration are associated not with lower but with greater zein degradation. These findings support the notion that zein degradation in whole plant maize silage is driven primarily by proteolysis during the long-term storage.

Acknowledgments

This research was financed by the Ministry of Science, Education and Sports of the Republic of Croatia (Research Grant 178-1780496-0368, 'Nutritional, antioxidant and prebiotic attributes of corn for domestic animals'). The authors would like to thank Siniša Škvorc and Goran Kiš for their contributions.

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