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PASTURE AND FORAGE UTILIZATION

Effects of easy soluble carbohydrate and bacterial inoculant supplementations on silage quality of frosted maize

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ABSTRACT. The present experiments were conducted to assess the effects of barley, *Lactobacillus* bacteria and lactic acid bacteria (LAB) and enzyme mixture supplementations on chemical composition and some quality parameters of naturally frosted maize silage. The treatments were as follows; T1: Control (no supplementation, C); T2: barley 20 g kg^{-1}, B20; T3: barley 40 g kg^{-1}, B40; T4: 1.5 g ton^{-1}, a mixture of LAB; and T5: 2 g ton^{1}, a mixture LAB+enzyme, LEN. Barley supplementation to frosted maize increased dry matter (DM) ($p < 0.01$). The pH value was higher in LAB group ($p < 0.01$). Treatments supplemented LAB and LEN significantly reduced crude protein contents ($p < 0.001$). Lactic acid contents decreased in all treatments as compared to control group and ranged between 2.46 and 5.40% DM ($p < 0.05$). The metabolisable energy content was significantly higher in B40 than in B20, LAB and LEN groups. Mean Fleig points showed with an "excellent" quality silage. In conclusion, supplementation of barley, LEN and inoculation of LAB to frosted maize had a good quality silage. The LEN supplementation may be used for efficient ensiling of frosted maize due to their ability to keep its quality in low DM crop maize silage.

Keywords: cold; plant killing; silage additives; organic acids; fleig point.

Efeitos da suplementação por caboidrato solúvel e de inoculantes bacterianos sobre a qualidade da silagem de milho coberto por geada

RESUMO. Os presentes experimentos foram conduzidos para avaliar os efeitos da cevada com a bactéria *Lactobacillus* e bactéria ácido lática (LAB) e suplementos de mistura enzimática sobre a composição química e alguns parâmetros de qualidade da silagem de milho cobertos por geada. Os tratamentos foram os seguintes; T1: Controle (sem suplementadão, C); T2: cevada 20 g kg^{-1}, B20; T3: cevada 40g kg^{-1}, B40; T4: 1,5 g ton^{-1}, uma mistura de LAB; e T5: 2 g ton^{-1}, com mistura de LAB+enzima, LEN. A suplementação de cevada para o milho aumentou a matéria seca (MS) ($p < 0,01$). O valor do pH foi maior no grupo LAB ($p < 0,01$). Os tratamentos complementados com LAB e LEN reduziram significativamente os conteúdos de proteínas brutas ($p < 0,001$). O teor de ácido láctico diminuiu em todos os tratamentos em comparação com o grupo controle e variou entre 2,46 e 5,40% de DM ($p < 0,05$). O teor de energia metabolizável foi significativamente maior em B40 do que nos grupos B20, LAB e LEN. Os pontos médios do Fleig mostraram uma silagem de "excelente" qualidade. Em conclusão, a suplementação por cevada, LEN e LAB em milho fosco apresentou silagem de boa qualidade. A suplementação com LEN pode ser utilizada para ensilagem eficiente de milho coberto por geada, devido a sua capacidade de manter sua qualidade em baixa MS.

Palavras-chave: frio; morte de plantas; aditivos de silagem; ácidos orgânicos; ponto fleig.

Introduction

Maize is the major silage crop used in majority of intensive ruminant production systems of many countries throughout the world (Kaplan et al., 2016). Location and weather conditions may allow maize culture twice a year in Turkey. When it is planted as a second crop, plants may be exposed to seasonal low temperatures. Early frosts during the grain-filling period can cause serious losses in maize yield (Bagg, Stewart, & Wright, 2013). A killing frost can occur when the air temperature drops to 0°C for 4 to 5 hours or to -2°C for 5 to 10 minutes (Narasimhalu, White, & McRae, 1986; White, Winter, & Kunelius, 1976). This low temperature can kill the entire maize plant, damaging leaves, stalk, ear shank and husks (Dekalb extension). Symptoms will start to show up about 1 to 2 days after a frost, but it often takes 5 to 7 days to assess the damage. Frost damage symptoms are generally encountered as water-soaked leaves or as plants turned into light yellow-brown color mixture. There are many questions about whether or not these plants should be ensiled, or when should be harvested (soon or late after freezing) (Aoki, Oshita, Namekawa, Nemoto, & Aoki, 2013; Morales et al., 2011). Severe impacts on grain quality may occur during frosts at the dough stage, with moderate impacts at the early dent stage and only minor impacts once corn kernels have reached half milk line (Khan, Yu, Ali, Cone, & Hendriks, 2015). However, although farmers are aware of low quality of silage made from frosted maize plants, they can improve silage quality with some additives such as starch or inoculants (Kutlu & Çelik, 2005). Microorganism growth and fermentation requires sufficient easily soluble carbohydrates such as barley in ensiling process.

Cold weathers may result in abnormal maize plants for silage, kernel moisture content can make milk-line quite variable and invisible (Bagg et al., 2013). Therefore, additives can be used based on kernel moisture levels to improve silage quality. Lactic acid bacteria and fermentation play a significant role on pH and silage quality. Calder, Langille, and Nicholson (1977) indicated that heavily frosted maize plants loose dry matter (DM) and feeding value when they left standing in the field for a long period of time; however, maize plants harvested soon after freezing were not seriously affected. Although corn forage harvested at boot stage has shown similar nutritional values with others harvested at later maturity stages, DM contents are lower being compensated by a greater regrowth during the second cut within the season (Morales et al., 2011). On the other hand, although high cutting of corn plants increase the NDF and ADF digestibility (and probably total digestible nutrients content), the nutritional losses per unit area due to high cutting was much less when corn forage was harvested at a later stages and without any detrimental impact on its fermentative quality and aerobic stability (Aoki et al., 2013).

Although there are several studies about silage quality, the information about the effect of freezing on silage quality and nutritive value is quite insufficient. Previous studies mostly focused on improving maize silage quality under normal environmental conditions, accurate moisture and maturation stages. Therefore, this study was conducted to investigate the effects of different additives, such as barley (as starch source), lactic acid bacteria (LAB) and LAB+enzymes mixture (LEN) (as inoculants) on quality attributes of frosted maize silage.

Material and methods

Experimental design

Maize was planted on July 18, 2013 to get silage material, Plants were grown over 5 ha land area of Erciyes University Agricultural Research and Application Center with standard irrigation and fertilization practices until October 7, 2013. Before the harvest, the environmental temperature fell to"-1" for 8 hours and the upper leaves of the maize plants turned to straw-colored. Plants were harvested with a conventional silage machine (Çelikel Challenger, Turkey) and choppedin1.5 - 3 cm pieces. Then samples were taken for analyses. The samples were tightly filled into glass jars (2 liters in volume) with 6 replicates and kept at room temperature (about 20-26°C) for 90 days. The treatment groups were arranged as follows:T1: Control (C, without any supplementation), T2: supplemented with barley (20 g kg^{-1} - B20), T3: supplemented with barley (40 g kg^{-1} - B40), T4: inoculated with LAB (1.5 g ton^{-1}, a mixture of LAB consisting of *Lactobacillus plantarum* and *Enterococcus faecium* applied at a rate of 6.00 log10 cfu LAB g^{-1} of fresh material, Pioneer 1174, USA),and T5: inoculated with LAB+enzyme mixture 2 g ton^{-1}(LEN, *Lactobacillus plantarum* bacterium (6.00 log10 cfu g^{-1}) and cellulose (150000 CMCU kg^{-1}) and amylase (200000 SKB kg^{-1}) enzymes, Silaid WS™, Global Nutritech Co., USA). Grinded barley was mixed and LAB and LEN were dissolved in 20mL water and sprayed over the chopped maize samples.

Chemical analyses

At the end of the 90 days ensiling period, the silages were sampled for chemical and other analyses. The pH measurements were taken from 25 g of silage samples through placing in a beaker, adding 100 ml of distilled water and blending for 5 minutes. Water was filtered and silage pH was measured (Akyildiz, 1986). The dry matter (DM), crude ash (CA), crude protein (CP), crude fiber (CF) and ether extract (EE) were determined in accordance with the methods specified in Association of Official Analytical Chemist (AOAC, 2000). Neutral detergent fiber (NDF) was analyzed with heat-stable amylase and without Na-sulfite, acid detergent fiber (ADF) was determined according to the sequential method of Van Soest, Robertson, and Lewis (1991) in an ANKOM fiber analyzer ($ANKOM_{220}$ Technology, Macedon, NY, USA) and expressed inclusive of residual ash. Hemicellulose was defined as NDF – ADF. The fleig point (FP) was calculated with the equation:

$$FP = 220 + (2 \times DM\% - 15) - 40 \times pH.$$

The Total digestible nutrients (TDN) were calculated according to the equation proposed by Chandler (1990), where:

$$TDN\% = 105.2 - 0{,}68 \times NDF\%.$$

The Non-Fiber Carbohydrates (NFC) were calculated by the equation proposed by Weiss, Conrad, and Pierre (1992):

$$NFC\% = 100 - (NDF\% + CP\% + EE\% + CA\%).$$

Total carbohydrates (TC) were determined according to Sniffen, O'Connor, Van Soest, Fox, and Russell (1992) with the equation:

$$TC\% = 100 - (CP\% + EE\% + CA\%).$$

The metabolisable energy (ME) was calculated by the equation proposed by Robinson, Givens, and Getachew (2004):

$$ME = 14.03 - (0{,}01386 \times CF\%) - (0.1018 \times CA\%).$$

To determine water soluble carbohydrate (WSC) content, liquid extractions were prepared with 40 g silage. Samples were placed into a beaker, 360 mL distilled water was added and mixed in a blender. The resultant slurry was filtered through Whatman 54 filter paper and then centrifuged. Samples were stored at -20°C until the analyses. The WSC of samples were determined by phenol sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The lactic acid (LA) of silages were determined by Lepper's methods according to Akyildiz (1986) and acetic, propionic and butyric acid were determined in a gas chromatograph with a capillary column (30 m × 0.25 mm × 0.25 μm, Restek), in a Shimadzu GC-2010+ (Kyoto, Japan) gas chromatograph equipped with FID, over a temperature range of 45–230°C.

In-vitro dry matter digestibility was determined by Adesogan (2005). The rumen fluid was obtained from a cattle slaughtered at a local slaughterhouse. All treatment groups were analyzed with the same rumen fluid. After incubation (Daisy^{II} incubator, ANKOM Technology, USA), sealed bags were extracted sequentially with ND (without either alpha-amylase or sodium sulphite) to determine the amount of undigested NDF remaining in each bag.

Statistical analysis

Data were analyzed using the general linear model procedure of the SPSS Inc. (2008). Differences between means were determined using the Duncan's multiple range tests with a 5% level of probability. The results of statistical analysis were shown as mean values and standard error of the means (SEM).

Results and discussion

The effects barley, LAB and LEN supplementations on chemical composition of frosted maize silage are shown in Table 1. Various additives have been used to get better quality silage (Akyildiz, 1986). Barley supplementation (20 and 40 g kg^{-1}) increased DM content of silages as compared to the control, LAB and LEN groups ($p > 0.001$). The LAB group showed lower DM values than LEN group, which might be explained by higher LA production ($p < 0.05$) and consumed acetic acid (AA) and bacterial inoculants (Table 2). When maize plants were exposed to severe cold weathers, leaves are damaged and it is difficult to accurately estimate the whole-plant moisture from kernel milk-lines. Corn silage quality can significantly be influenced by the whole plant moisture and maturity of the maize at harvest (Bagg et al., 2013). In present experiments, DM content of silage was close to desired levels in maize being appropriate to silage. White et al. (1976) reported the highest *in vitro* DM digestibility and *in vitro* digestible DM yields and the lowest cellulose content at the time of the first frost. Also nitrogen (N) and potassium (K) contents of the whole plant decreased significantly with the time after frost. The total amounts of N, phosphorus (P) and K also decreased in maize plants harvested after frost.

Table 1. Effects of barley, LAB and LEN supplementation on crude protein, ether extract, crude ash, crude fiber, hemicellulose, acid detergent fiber (ADF), neutral detergent fiber (NDF) of frosted maize silage.

Items	Treatments					SEM	P
	Control	B20	B40	LAB	LEN		
Dry matter, %	30.77^{bc}	33.18^{a}	32.88^{a}	29.95^{c}	31.53^{b}	0.28	***
pH	4.14^{bc}	4.24^{b}	4.14^{bc}	4.50^{a}	4.08^{c}	0.03	***
Crude protein,%	9.63^{a}	9.01^{ab}	10.07^{a}	8.92^{ab}	7.79^{b}	0.22	***
Ether extract,%	1.13	1.12	1.17	1.17	1.18	0.02	n.s
Crude Ash, %	7.47^{a}	7.84^{a}	6.35^{b}	7.88^{a}	7.31^{a}	0.16	*
Crude fiber,%	25.54	26.46	26.52	26.2	26.06	0.31	n.s
Hemicellulose, %	17.01	16.94	13.76	11.89	10.73	0.89	n.s
ADF,%	29.6	29.52	28.45	31.45	33.19	0.69	n.s
NDF,%	46.61	43.27	45.38	43.33	43.93	0.64	n.s

Control: no additive; B20 and B40: 20 and 40 g kg^{-1} grinded barley respectively, LAB: *Lactobacillus plantarum* and *Enterococcus faecium* 1.5 g ton^{-1}; and LEN: *Lactobacillus plantarum*, cellulose and amylase enzymes 2 g ton^{-1}. ^{a,b,c}: Values with different superscript in the same line differ significantly *: $p < 0.05$; P: probability; SEM: standard error of means. n.s: non-significant.

In this study, the pH values of silage samples varied between 4.08 - 4.50 ($p < 0.001$) (Table 1). The pH value of LAB-supplemented group was higher than the other groups. The lowest pH was found in the LEN group. Mohammadzadeh, Khorvash, Ghorbani, and Yang (2012) reported that there was a tendency to have higher pH values in silage inoculated with LAB and prepared from frost-killed maize. However, from a practical point of view, the most important thing that must occur in silage preparation is rapid drop in pH value. During the ensiling process, lactic acid bacteria utilize water-soluble carbohydrates to produce LA. Then, acidity is increasing and subsequently pH is decreasing in the silage (Kung & Shaver, 2001). These pH results were higher than the recommended levels (between 3.5 - 4.2), but similar to those found under practical conditions (Konca, Alçiçek, & Yaylak, 2005). However, Narasimhalu et al. (1986) reported that post-frost silages were drier with pH values higher than the other silages. Depending on the plant material used to prepare silage, pH can vary between 5-6 and may decrease to 3.6-4.5 after acid production. A quick reduction in pH will help to reduce the breakdown of protein in silage through inactivation of plant protease.

Table 2. Effect of barley, LAB and LEN supplementation on water soluble carbohydrates (WSC), lactic acid (LA), acetic acid (AA), propionic acid (PA) and butyric acid (BA) as a percentage of DM.

Items	Treatments					SEM	P
	Control	B20	B40	LAB	LEN		
WSC, % DM	2.72	3.86	3.87	4.16	4.51	0.206	n.s
LA, % DM	5.40^{a}	2.46^{b}	3.71^{b}	3.89^{b}	3.27^{b}	0.259	*
AA, %DM	4.69	3.16	4.26	4.84	4.42	0.366	n.s
PA, %DM	0.13	0.18	0.19	0.2	0.33	0.021	n.s
BA, %DM	0.011	0.019	0.031	0.046	0.118	0.023	n.s

Control: no additive; B20 and B40: 20 and 40 g kg^{-1} grinded barley respectively, LAB: *Lactobacillus plantarum* and *Enterococcus faecium,* 1.5 g ton^{-1}; and LEN: *Lactobacillus plantarum*, cellulose and amylase enzymes, 2 g ton^{-1}. ^{a,b,c}: Values with different superscript in the same line differ significantly *:$p < 0.05$; P: probability; SEM: standard error of means. n.s: non-significant.

The CP values of silage samples from frosted maize ranged between 7.79 and 10.07 % (Table 1). The 40g kg^{-1} barley group showed the highest CP contents (together with control group) and the LEN group had the lowest ($p < 0.001$). Compared to control group, treatments supplemented with LAB and LEN significantly reduced CP contents which probably indicate a higher proteolysis in the silage of these two groups. Similar results were reported by Basso et al. (2012) indicating that LAB inoculates significantly decreased CP concentrations of maize silage. In present study, crude ash values were similar in all groups except for B40 group which shows a lower value ($p < 0.01$). However, the values obtained herein were similar to those reported by Konca et al. (2005) for normal maize silage. The ether extract, crude fiber, hemicellulose, ADF and NDF values of treatment groups were not different from each other ($p > 0.05$) (Table 1). Gurbuz and Kaplan (2008) reported similar ADF and NDF ratios for pure maize silage. Mohammadzadeh, Khorvash, and Ghorbani (2014) reported that DM, NDF, CP and WSC ratios were not influenced from frosting. In contrast, frosting increased yeast population on crop. Narasimhalu et al. (1986) reported that detergent fiber ratios were higher in the post-frost than pre-frost silages. Although ADF and NDF contents did not exhibited any significant differences between treatment groups, higher ADF values were observed in LEN group. In contrast, higher NDF values were observed in control group. Increases in fiber concentration can be explained by differences in the amount of DM losses that happen during the fermentation process. The enzyme supplementation to silage had been practiced as a method to degrade cell walls and subsequently improve the digestibility of silage fiber (McDonald, 1981). The higher fiber values may be achieved through combined effect of lower DM recovery and reduced hydrolysis of hemicellulose in frosted silages treated with LAB or enzyme groups (Basso et al., 2012). However, it was not expected that LAB or LEN treatment decreased NDF contents (Guan, Driehuis, & Wikselaar, 2003).

With regard to inoculants, there were some differences in fermentation quality between the control and the biological additive-treated silages. Table 2 shows the effects of starch and inoculant supplementations on WSC, LA, AA, PA BA (as a % DM) of frosted maize silage. It is known that WSC, LA, AA and BA concentrations are important criteria to evaluate silage fermentation quality. Although there aren't any significant effects of supplements on WSC concentration of silage, a trend to higher WSC values were observed in LAB and LEN-supplemented groups than the control group. In general, maize silage harvested at the 2/3 milk-line maturity stages has lower WSC levels (3-10%) than the barley silage (10-20%) and had larger populations of epiphytic lactate-producing bacteria (Muck, 2010). One of the objectives of silage additives is to enhance efficient fermentation through stimulation of lactic acid production. As compared to control treatment, LA contents decreased in all treatments in this study and ranged between 2.46 and 5.40% DM ($p < 0.05$). Although the differences were not significant, higher AA values were observed in LAB group, which can be inferred that *Lactobacillus* was more effective in improving LA and AA than the mixture *Lactobacillus* plus enzymes.

The ether extract, crude fiber, HEM, NDF and ADF% values of the treatment groups were not significantly different ($p > 0.05$). Mohammadzadeh et al. (2014) reported that in the frost-killed corn DM, NDF, CP and WSC ratios were not affected by frosting. However, population of lactic acid producing bacteria decreased in response to frosting. In contrast, frosting increased yeast population on crop. Narasimhalu et al. (1986) reported that detergent fiber ratios were higher in the post-frost than pre-frost silages. Calder et al. (1977) showed that corn which has been heavily frozen will lose DM and feeding value if left standing in the field for a period of time; however, corn harvested soon after freezing is not as seriously affected. Mohammadzadeh et al. (2014) investigated the differences in fermentation quality of control and biological additive-treated silages.

The effects of barley, LAB and LEN supplementation on total digestible nutrient (TDN), non-fiber carbohydrate (NFC), total carbohydrate (TC), metabolic energy (ME) and fleig point (FP) are presented in Table 3.

Table 3. Effects of barley, LAB and LEN supplementation on total digestible nutrient (TDN), non fiber carbohydrate (NFC), total carbohydrate (TC), metabolisable energy (ME) and fleig point (FP).

	Treatments					SEM	P
Items	Control	B20	B40	LAB	LEN		
TDN,%	85.07	85.13	85.86	83.82	82.63	0.47	n.s
NFC, %	36.28	38.59	38.75	39.26	39.96	0.74	n.s
TC, %	82.89	81.86	84.64	82.08	83.89	2.33	n.s
ME, MJ kg^{-1} DM	13.01^{ab}	12.83^{b}	13.20^{a}	12.83^{b}	12.90^{b}	0.23	**
Fleig point	101.13^{a}	101.65^{a}	105.01^{a}	84.90^{b}	105.02^{a}	1.61	**
IVDMD	74.18	76.89	74.89	72.52	72.86	0.239	n.s

Control: no additive; B20 and B40: 20 and 40 g kg^{-1} grinded barley respectively, LAB: *Lactobacillus plantarum* and *Enterococcus faecium,* 1.5 g ton^{-1}; and LEN: *Lactobacillus plantarum*, cellulose and amylase enzymes, 2 g ton^{-1}. ^{a,b,c}: Values with different superscript in the same line differ significantly *: $p < 0.05$; P: probability; SEM: standard error of means. n.s: non-significant.

There were not any differences between the control and the supplemented groups in terms of TDN, NFC and TC content of the silage ($p > 0.05$). However, the ME content was significantly higher in B40 than in B20, LAB and LEN groups. In this study, the average mean Fleig points of frosted maize silages ranged between 84.90 and 105.02. The lowest values were observed in LAB treatments compared to the rest of the groups ($p < 0.01$). According to the Fleig point system, these values coincide with "excellent" quality silage, giving to farmers the possibility to improve quality of frozen maize silage. Similar to present study, Calder et al. (1977) reported that corn ensiled after frost showed a lower lactic acid content (and in it's the less desirable form) than those harvested before frost. On the other hand, the maize plants that had been frosted before the harvest time should exhibit a decrease on DM digestibility, energy and protein content. However, all this deleterious effects on nutritional value should be minimized through harvesting corn as soon as possible after frost to avoid higher moisture loss in the crop and the consequent problems (Calder et al., 1977). The search for silage additives to get a faster pH decrease should favor the development of some specific bacteria such as *L. plantarum* and *L.casei* (Montville & Matthews, 1997). Kung and Shaver (2001) working on maize silage inoculated with *L. buchneri* reported a reduced LA concentration and promoted energy and DM recovery. However, a very low AA concentration adversely affects the aerobic stability of the silage. In this study, the acetic acid concentration in all the treated silages ranged from 3.16- to 4.84 % DM, as being considered well enough to maintain the aerobic stability of the silage.

Finally, regarding the fermentation characteristics and aerobic stability of the silages, both should be improved by inoculation of homofermentative and heterofermentative LAB to maize silage (Hu, Schmidt, McDonell, Klingerman, & Kung, 2009). Muck (2010) reported that forages inoculated with homofermentative LAB before ensiling showed higher LA concentrations than heterofermantative LAB. Mohammadzadeh et al. (2014) evaluated the early and late effects of multispecies LAB inoculants on biochemical characteristics of corn silages at different maturity stages and reported that the maturity stage of maize crops had an impact on its chemical composition and microbial characteristics influencing the fermentation characteristics. However, LAB inoculants will improve only the aerobic stability of mature crops after a long-time ensiling.

Conclusion

Silage prepared by supplementation of barley, LEN and inoculation of LAB to frosted maize had a good quality. Furthermore, LEN supplementation exhibited excellent quality silage. Therefore, LEN supplementation may be used for efficient ensiling of frosted maize due to their ability to keep its quality in low DM maize silage.

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