The effects of *Lactobacillus plantarum* and *Propionibacterium acidipropionici* on corn silage fermentation, ruminal degradability and nutrient digestibility in sheep

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Summary

The chemical composition, *in situ* ruminal degradability coefficients of dry matter (DM), and nutrient digestibility in sheep were evaluated for corn silage (CS) treated (fresh weight basis) with different levels of a commercial bacterial inoculant (I) as follows: (1) untreated CS (control); (2) CS + I at half of the recommended level; (3) CS + I at the recommended level (3×10^{10} colony forming units per g of fresh forage) and (4) CS + I at two-fold recommended level. The inoculants (Lallemand, France) consisted of *Lactobacillus plantarum* and *Propionibacterium acidipropionici*. Whole- plant corn was ensiled for 60 days in plastic polyethylene bags. Also, three laboratory silos (70 g capacity) for each treatment were sampled on days 2, 3, 4 and 60 to study the pH changes. The silages underwent rapid fermentation and were well-preserved. The pH value decreased to 4.0 on day 2 and was the lowest for treatment 2 on day 60 after ensiling compared with other treatments. Treatment 2 had higher ($P<0.05$) contents of crude protein (CP), residual water soluble carbohydrates (WSC), lactic acid, acetic acid, propionic acid, and total acids, but lower ($P<0.05$) butyric acid than treatment 3, and also higher acetic and lower butyric acid levels than other treatments. No traces of ethanol were detected in any of the silages. The acid detergent fiber (ADF) content of treatments 1 and 4 was lower ($P<0.05$) than others. Only ether extract (EE) digestibility was affected by these treatments which was higher for treatments 1 and 4 ($P<0.05$). The DM recovery for treatment 1 was lower than others. Effective DM degradability was slightly higher for treatment 4 and fraction "a" was significantly ($P<0.05$) lower for treatments 1 and 3 compared with treatment 4. The results indicated that application of this inoculant at half of the recommended level was more effective to enhance the aerobic stability of silages due to higher acetic and propionic acid production which have antimycotyic properties. The decreased cost associated with this level of inoculant might be economical for farmers in warm climates as to encourage its use as an additive for silage making.

Key words: Corn silage, Propionic acid bacteria, Lactic acid bacteria, Digestibility

Introduction

The main objective of adding additives to silages is to obtain lactic acid fermentation that results in a well-preserved silage (McDonald, 1981) because of preventing secondary fermentations and decreasing butyric acid production. The biological additives are more preferred because they are safe and easy to use, are not corrosive to machinery, do not pollute the environment and are regarded as natural products.

Several studies have shown the beneficial effects of bacterial inoculation on corn silage preservation (Higginbotham *et al*., 1998; Ranjit and Kung, 2000). Bacterial/Inoculants stimulate lactic acid production with low pH. Therefore, feed intake, dry matter (DM) and organic matter (OM) digestibilities are increased, resulting in a better animal performance (Chamberlain, 1982; Wheeler and Mulcahy, 1989; Havillan and Kaiser, 1992; Bolsen *et al*., 1996). Microbial additives increased silage quality, nutrient digestibility and net

energy for lactation, and reduced protein degradation (Ilakova *et al*., 1998). However, DM digestibility of treated silages was not affected in some studies (Kung *et al*., 1993; Rooke *et al*., 1998). The growth inhibition of undesirable bacteria is associated with the level of lactic acid production following ensiling, which depends on the initial population of lactic acid bacteria (LAB) and substrate availability at ensiling (McDonald *et al*., 1991). Bacterial inoculants generally increase lactic acid level and reduce silage pH, acetic and butyric acid levels in silages which indicate good fermentation (Sanderson, 1993; Kennedy, 1994; Kung *et al*., 1987). Addition of bacterial inoculant containing homofermentative LAB improved the quality and aerobic stability of grass silage (Wrobel and Zastawny, 2004). With corn silage, it is shown that the inoculants of LAB improved the fermentation quality, increased WSC and lactic acid contents and decreased acetic acid, butyric acid and ammonia-N (Pahlow and Hoing, 1994; Jatkauskas and Vrotniakiene, 2004). However, such silages have low aerobic stability (Weinberg *et al*., 1993; Filya *et al*., 2002) due to insufficient volatile fatty acid (VFA) production for protecting the silage against aerobic yeasts and moulds (Moon, 1983). High levels of residual WSC, combined with high lactic acid concentrations and insufficient production of protective VFA in the silages inoculated with homofermentative LAB were associated with aerobic spoilage (Weinberg *et al*., 1993). Propionic acid bacteria can ferment sugars and lactate to acetate and propionate, which inhibit the growth of yeasts and moulds in silage (Woolford, 1975; Moon, 1983). The DM, ADF, WSC, total N, neutral detergent fiber (NDF), pH, lactic acid and ammonia-N contents of corn silage containing 22.6% DM were not affected by treatment with *propionibacteria* and LAB during 90 d fermentation (Higginbotham *et al*., 1998). In recent years, marked changes have been made to the formulation and recommended application rates of additives containing propionic acid. In a study by Higginbotham *et al*. (1996), application of *propionibacteria* at 1×10^6 cfu/g corn plant decreased the silage pH after 30 days of fermentation, however, no differences were noted for concentrations of WSC or lactic, acetic and propionic acids. No marked differences in chemical composition were observed when pearl millet and corn silages were inoculated with *Prop. shermanii* or *Prop. shermanii* plus LAB at ensiling (Weinberg *et al*., 1995).

The objective of this study was to investigate the effects of a commercial bacterial inoculant applied at the time of ensiling whole-plant corn on the fermentation characteristics, rumen degradability, and nutrient digestibility in sheep. This product is available in Iran but its high price prevents farmers to use it as a silage additive. Therefore, a comparison between the effects of half and twice of its recommended level by the manufacturer was also made.

Materials and Methods

Silage preparation

Whole-plant corn was harvested at the early dent stage of maturity with approximately 30% DM from a corn field in the College of Agriculture, Shiraz University, Iran. The commercial inoculant (LALSILMSOI, Lallemand SA, Saint-Simon, France) contained *Lactobacillus plantarum* MA18/5U and *Propionibacterium acidipropionici* MA26/4U. Four treatments were used in the experiment: 1) Untreated corn silage (CS) as control; 2) CS + I at half of the recommended level; 3) CS + I at the recommended level and 4) CS + I at two-fold recommended level. The recommended level was 3×10^{10} colony forming units (cfu)/g of the fresh forage, which was applied according to the manufacturer's recommendation with no verification. In order to prepare each treatment, sufficient chopped forage was placed on a polyethylene sheet and the specified solution of inoculant was sprayed, followed by thorough mixing. The same volume of water which was used to solubilize the additive, added to the control silage to maintain equal moisture levels. Three samples from the pre-ensiled forage and treated silages were placed on ice and transported to the laboratory for chemical analysis. Dark

polyethylene bags were packed with 20 kg of the corn forage. Ten silo bags were used for each treatment, kept indoors and opened after 60 days of ensiling. At the same time, 12 mini-silos (70 g capacity plastic cylinders fitted with a Bunsen rubber valve lid that enable gas and seepage to release) were filled with corn forages and triplicate silos were opened at 2, 3, 4 and 60 days after ensiling to study pH changes (time course of fermentation). At the end of the ensiling period, a 500 g silage sample from each silo bag was taken for chemical analysis and determination of fermentation acids. Dry matter losses during the fermentation and storage phases were estimated by weighing the mini silos before ensiling and again on day 60 post-filling.

Chemical analysis

Chemical composition of the fresh corn forage, silages, and feces were determined following the procedures of AOAC (2000). Neutral and acid detergent fibers were determined according to the Goering and Van Soest method (1970). The pH of each sample was determined in triplicates using 25 grams wet material added to 100 ml of distilled water. After homogenizing in a blender for 10 min, the pH was determined using a digital pH meter (Polan *et al*., 1998). Then, an aliquot of the homogenized sample was strained through 2 layers of cheesecloth, and the liquid fraction was centrifuged at 2000 × g for 20 min and stored frozen for organic acid analysis. Water-soluble carbohydrates were determined by the phenol sulfuric acid method (Dubois *et al*., 1956). The silage organic acids were determined by gas chromatography (Crompack, Model CP 9002, The Netherlands) as described by Playne (1985).

Ruminal DM degradability

Rumen degradability was estimated *in sacco* (Orskov and McDonald, 1979). The dry samples were ground using a grinder with a 2 mm sieve. Approximately, 5 g DM of each sample was transferred into polyester bags (12 × 19 cm) with 50 μm pore size. Four bags for each treatment and inoculation time were incubated in the rumen of two fistulated Sistani bulls (450 kg BW) for 2, 4, 8, 12, 24, 48 and 72 h. The cattle were fed a diet consisting of 90% mixture of wheat bran and alfalfa hay (50/50) and 10% pistachio hulls. The ration was fed in equal portions every 12 h to maintain a relatively stable ruminal environment.

Four bags were also washed with cold tap water to estimate zero time washouts. After each incubation time (including the zero time), the bags were removed and hand-washed with cold water until the water remained clear. Then, samples were oven-dried at 55°C until a constant weight was achieved for each sample before determination of DM disappearance. Loss of DM at various incubation intervals was fitted to the non-linear equation $p = a+b\ (1-e^{-ct})$, in which p is the amount degraded at time, "a" is the fraction that is soluble or immediately degraded, "b" is the fraction that is potentially degradable but insoluble, and "c" is the fractional rate constant at which the fraction "b" will degrade per hour.

Digestibility experiment

Sixteen Mehraban rams (mean BW 39.9 ± 2.0 kg) were divided into three equal groups with similar mean body weight (BW) and similar variations between lambs within group. Lambs were housed individually in crates. Each experimental period was for 24 days, which included 16 days of adaptation to the experimental diets followed by 8 days collection periods, during which separate collection of total feces was made. They had free access to fresh water. Lambs were fed diets containing 30% of a pelleted commercial concentrate mix (DM = 98.0%, NDF = 56.5%, ADF = 10.9% and CP = 16.0%) and 70% of the experimental silages. They were fed diets DM based on 4% BW in two equal meals at 8:00 a.m. and 16:00 p.m. Each day, 10% of the daily fecal output was sampled for each sheep and kept frozen until chemical analysis.

Statistical analysis

The obtained data for the DM degradability coefficients (a, b and c) were analyzed by one way analysis of variance, and other data were subjected to analysis of

variance using general linear model procedure (SAS, 1996). Mean separation was performed by the Duncan's multiple range test with a level of significance set at 5%.

Results

The chemical composition and pH of the fresh and ensiled corn forages are shown in Table 1. Concentrations of OM, ether extract (EE), and NDF were not significantly different amongst the silages. Treatment 2 had the lowest pH. Treatments 2 and 3 had higher CP and ADF levels. The lowest and highest DM contents were found for treatments 1 and 4, respectively, with intermediate values for treatments 2 and 3.

All levels of the inoculant improved DM recovery as compared with the control (Table 2). Acetic acid production as well as total acid were decreased in treatments 3 and 4. Lactic and propionic acid production were decreased but butyric acid production was increased in treatment 3. Treatment 4 had significantly a higher lactate-to-acetate ratio (Table 2). Table 3 shows the pH changes of the laboratory silos at various days after ensiling. The pH values on day 60 for treatments 2 and 3 were significantly lower than the values for the control and treatment 4.

No significant differences were found among treatments for the degradability of DM in fractions "c" and "a+b" (Table 4). Treatment 4 had higher "a" fraction than treatments 1 and 3 with no significant difference between treatments 2 and 4. The digestibility coefficients of the nutrients were not significantly affected by the presence of inoculants, except for EE (Table 5). Digestibility of EE was notably lower for treatments 2 and 3 as compared with other treatments.

Discussion

The main objective of using additives

Table 1: The chemical composition (DM basis) and pH of fresh corn forage, control and inoculant-treated corn silages at 60 days post-ensiling in experimental silages

Materials	DM	CP	OM	EE	WSC	NDF	ADF	pH
Fresh forage	29.14	7.48	95.17	3.50	15.64	67.70	22.64	5.90
Treatments								
1	25.63^{c}	6.27^{b}	95.50^{a}	3.00^{a}	3.91^{ab}	63.37^{a}	23.80^{b}	4.22^{a}
2	27.21^{b}	7.07^{a}	95.50^{a}	2.00^{a}	4.71^{a}	63.72^{a}	26.67^{a}	4.01^{b}
3	27.91^{b}	6.40^{ab}	95.50^{a}	1.67^{a}	3.69^{ab}	67.66^{a}	26.60^{a}	4.22^{a}
4	29.73^{a}	6.14^{b}	95.50^{a}	2.00^{a}	3.31^{b}	68.52^{a}	22.46^{b}	4.24^{a}
SEM	0.39	0.21	0.35	0.41	0.35	2.06	0.84	0.02

Treatments: 1) control (untreated CS); 2) CS + I at half of the recommended level; 3) CS + I at the recommended level; 4) CS + I at two-fold recommended level. ADF: Acid detergent fiber, CP: Crude protein, DM: Dry matter, EE: Ether extract, NDF: Neutral detergent fiber, OM: Organic matter, WSC: Water-soluble carbohydrates. $^{a, b, c}$: Within each column, means with any common superscript(s) do not differ significantly (P>0.05)

Table 2: The fermentation characteristics (DM basis) and dry matter recovery (% of DM ensiled) of the inoculant-treated corn silages in laboratory silos

Items	Treatments				SEM
	1	2	3	4	
Acetic acid	2.18^{a}	2.23^{a}	1.32^{b}	1.07^{b}	0.14
Lactic acid	7.37^{a}	8.17^{a}	3.05^{b}	6.39^{a}	0.78
Propionic acid	0.245^{ab}	0.445^{a}	0.075^{b}	0.250^{ab}	0.070
Butyric acid	0.109^{b}	0.040^{b}	0.285^{a}	0.132^{b}	0.038
Total acids	9.90^{ab}	10.88^{a}	4.69^{c}	7.84^{b}	0.811
Lactic/acetic ratio	3.41^{b}	3.69^{b}	2.10^{b}	6.35^{a}	0.761
DM recovery	94.43^{b}	97.30^{a}	96.70^{a}	96.83^{a}	0.542

Treatments: 1) control (untreated CS); 2) CS + I at half of the recommended level; 3) CS + I at the recommended level; 4) CS + I at two-fold recommended level. $^{a, b, c}$: Within each row, means with any common superscript(s) do not differ significantly (P>0.05)

Table 3: Effects of inoculant-treated corn silages and time of post-ensiling on pH changes in laboratory silos

Days post-filling	Treatments				SEM
	1	2	3	4	
2	4.30[a]	4.15[d]	4.25[b]	4.18[c]	0.005
3	4.64[a]	4.42[c]	4.57[ab]	4.47[bc]	0.029
4	4.31[b]	4.37[a]	4.32[b]	4.25[c]	0.009
60	4.36[a]	4.01[c]	4.12[b]	4.31[a]	0.020

Treatments: 1) control (untreated CS); 2) CS + I at half of the recommended level; 3) CS + I at the recommended level; 4) CS + I at two-fold recommended level. [a, b, c]: Within each row, means with any common superscript(s) do not differ significantly (P>0.05)

when making silages is to obtain a type of lactic acid fermentation that results in well-preserved silages. It is generally believed that microbial inoculation of silages has positive effects on fermentation because of decreasing pH, acetic and butyric acid and increasing lactic acid levels (Kung *et al*., 1987; Anderson *et al*., 1989; Kennedy, 1994; Rooke *et al*., 1998). The growth inhibition of undesirable bacteria is associated with the level of lactic acid production during ensiling, which depends on the initial population of lactic acid bacteria and substrate availability (McDonald *et al*., 1991). The addition of inoculants at the recommended and two-fold recommended levels produced silages which had lower quality or similar to the control. The lower DM content of the control treatment after 60 days of ensiling might be due to more extensive fermentation (McDonald *et al*., 1991). The higher CP content in treatment 2 is indicative of less proteolysis (Chamberlain *et al*., 1990; Rooke *et al*., 1998). Silage residual WSC decreased with time post-filling and was higher for treatment 2 than treatment 4. This shows more production of WSC in treatment 2 that results in higher total acids of treatment 2 than treatments 3 and 4. The lower ADF content of treatments 1 and 4 might be due to increased cell-wall digestion during fermentation (Bolsen *et al*., 1996). These findings are not in agreement with the results of Weinberg *et al*. (1995) who reported no marked differences in chemical composition of pearl millet and corn silages inoculated with *Prop. shermanii* or *Prop. shermanii* plus LAB at ensiling. In the study of Higginbotham *et al*. (1996) application of

Table 4: Ruminal degradation parameters of dry matter for inoculant-treated corn silages

Items[£]	Treatments				SEM
	1	2	3	4	
a	39.29[b]	40.77[ab]	38.03[b]	42.94[a]	0.912
b	43.87[b]	45.72[ab]	47.73[a]	44.05[b]	1.02
c(h-1)	0.0509[a]	0.0515[a]	0.0412[a]	0.0709[a]	0.009
a+b	83.16[a]	86.48[a]	85.76[a]	86.99[a]	1.16
ED	85.36	88.77	88.14	89.19	

Treatments: 1) control (untreated CS); 2) CS + I at half of the recommended level; 3) CS + I at the recommended level; 4) CS + I at two-fold recommended level. [£] a: Fraction soluble in water, b: Fraction degraded at a measurable rate, c: The rate at which the "b" fraction is degraded, "a+b": Potential degradability, and ED: Effective degradability values at 0.05 per hour outflow rate. [a, b, c]: Within each row, means with any common superscript(s) do not differ significantly (P>0.05)

Table 5: Effects of inoculation on nutrient digestibility (%) of corn silages

Parameters	Treatments				SEM
	1	2	3	4	
Dry matter	66.80[a]	66.33[a]	68.59[a]	69.59[a]	1.32
Organic matter	68.65[a]	68.79[a]	70.52[a]	71.45[a]	1.25
Crude protein	70.08[a]	70.23[a]	65.20[a]	70.66[a]	1.69
Neutral detergent fiber	61.89[a]	61.04[a]	65.03[a]	66.12[a]	1.89
Acid detergent fiber	41.10[a]	44.76[a]	51.17[a]	43.98[a]	3.25
Ether extract	79.21[a]	48.19[b]	39.74[b]	74.10[a]	3.98

Treatments: 1) control (untreated CS); 2) CS + I at half of the recommended level; 3) CS + I at the recommended level; 4) CS + I at two-fold recommended level. [a, b, c]: Within each row, means with any common superscript(s) do not differ significantly (P>0.05)

propionibacteria at 1×10^6 cfu/g fresh corn plant decreased the pH after 30 days of fermentation compared with the control silage, but there were no differences between concentrations of WSC or lactic, acetic and propionic acids.

All silages went through a rapid fermentation phase and the pH dropped to 4.0 at 2 d post-filling (Table 3). The treatment 2 ($P<0.05$) had the lowest pH (4.15) and treatment 1 had the highest pH (4.30) value, and almost the same trend was noted on 60 days post-filling in the laboratory and experimental silages. The low pH value at day 60 (4.01) for treatment 2 can potentially minimize the growth of clostridia due to the low concentrations of butyric acid (McDonald, 1981). Pahlow and Hoing (1994) reported that the propionic acid production by *propionibacteria* ceased below pH 4.80 but the final pH value in the present experiment was around 4.20 for all silages. Propionic acid was produced in all silages and was the highest (0.445% DM) for treatment 2. Weinberg *et al*. (1995) reported that the growth of *propionibacteria* was not sustained under the ensiling conditions in pearl millet and corn silages which is in contrast to the findings of the present experiment. All fermentation acids were affected by the treatments. The concentration of acetic acid was significantly ($P<0.05$) higher for treatments 1 and 2 with no differences between treatments 3 and 4. Propionic acid bacteria can ferment lactate to acetate and propionate which have antimycotic properties that inhibit the development of yeasts and moulds upon aerobic exposure of silages (Huber and Soejono, 1976; Moon, 1983). Treatment 2 had numerically higher lactic acid content compared with treatments 1 and 4 which shows the synergistic effects of *L. plantarum* and propionic acid bacteria at half of the recommended level. Final lactic acid contents, except for treatment 3, were within the expected ranges for silages containing moisture greater than 60% (6-8% DM) (Higginbotham *et al*., 1998). The concentration of butyric acid was the highest ($P<0.05$) for treatment 3 and treatment 2 numerically had the lowest butyric acid content than other treatments. In the study of Weinberg *et al*. (1995) no butyric acid was detected in the corn silage treated with *Propiobacterium shermanii*. The higher total acid concentration in treatment 2 compared with treatments 3 and 4 shows the beneficial interaction between LAB and propionic acid bacteria, in contrast to the findings of Parker and Moon (1982). It has been reported that propionic acid bacteria produced metabolites that benefitted the growth of LAB (Parker and Moon, 1982) via producing vitamins and other cofactors that might increase silage fermentation (Bullerman and Berry, 1965; Hettinga and Reinbold, 1972). The higher propionic acid content in treatment 2 is not in agreement with the results of Weinberg *et al*. (1995) who applied a propionic acid bacteria inoculants to corn silage. The higher lactic, acetic and propionic acid levels of treatment 2 were reflected in higher total acid concentration. The higher content of fermentation acids in treatment 2 indicated that more fermentable substrate was available and the added bacteria degraded the cell-walls. The lowest DM recovery in treatment 1 is in contrast to the findings of Hegginbotham *et al*. (1996). This shows more efficient fermentation due to microbial inoculation compared with the untreated corn silage. Lactic/acetic ratio was higher for treatment 4, followed by treatment 2, which indicates more homofermentative fermentation in these two treatments (McDonald, 1981).

No differences were found among these treatments for the degradability of DM in fractions “c” and “a+b”. Treatment 4 had higher “a” fraction than treatments 1 and 3 with no significant difference between treatments 2 and 4 which shows higher soluble fractions with these two treatments that can provide more soluble nutrients for the rumen microbes. The fractions “c” and “a+b” and effective degradability (ED) were numerically higher for treatments 2 and 4. These findings are in agreement with the previous reports (Filya *et al*., 2002; Filya, 2003). Nutrient digestibility was not generally affected by the treatments, but EE digestibility was higher for treatments 1 and 4. These findings are in agreement with those of Raeth-Knight *et al*. (2007) for DM, CP and NDF digestibilities in dairy cows fed *Lactobacillus acidophilus* and *Propionibacteria freudenreichii*. Pahlow and

Hoing (1994) found that the inoculation of corn silage led to an improvement of silage quality and nutrients digestibility.

The addition of microbial inoculant containing a propionic acid bacteria plus *L. plantarum* produced well-fermented silages and did not affect nutrient digestibility but, application at half of the recommended level resulted in silages with higher lactic, acetic, propionic acids, total acids and lower butyric acid. Application at half of the recommended level could be more effective to enhance the aerobic stability of silages due to higher acetic and propionic acid production which have antimycotyic properties. The decreased cost associated with this level of inoculant might be economical for farmers in warm climates (Ashbell *et al*., 2002) as to encourage its use as an additive for silage making.

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