

The effects of *Lactobacillus plantarum* on chemical composition, rumen degradability, *in vitro* gas production and energy content of whole-plant corn ensiled at different stages of maturity

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Summary

This study was carried out to determine the effects of *Lactobacillus plantarum* on chemical composition and nutritive value of whole-plant corn (WPC) ensiled at different stages of maturity. Based on local routine practice, WPC was harvested at three stages of maturity as follows: (1) two weeks before routine harvesting time; (2), one week before routine harvesting time and (3), routine harvesting time. Bacterial inoculant (Ecosyl) was used as homofermentive lactic acid bacteria. The inoculant was applied at the recommended level of 1×10^5 CFU/g of fresh forage which was ensiled for 25 days in plastic polyethylene bags. Three untreated silages were prepared for each harvesting time and considered as controls (C). At the end of the ensiling period, all silages were subjected to chemical analysis, DM degradability and *in vitro* gas production. All silages underwent good fermentation and pH values decreased to below 4. WPC ensiled one week before routine harvesting time and treated with bacterial inoculant, had the lowest pH, ADF ($P < 0.05$) content and the highest CP content, total gas production, IVOMD, DOMD ($P < 0.05$), ME ($P < 0.05$), fraction "a" ($P < 0.05$) and ED ($P < 0.05$) compared with other treatments. The results indicated that application of bacterial inoculant (*Lactobacillus plantarum*) at the recommended level to WPC harvested at one week before routine harvesting time was more effective in enhancing chemical composition and nutritive value of silages, and provide a well-preserved and high nutritive value feedstuff for ruminants.

Key words: Bacterial inoculant, Corn silage, Stage of maturity, Nutritive value, *In vitro* degradability

Introduction

Whole-plant corn (WPC) is the major crop ensiled in Iran and plays an important role in supplying digestible fiber and energy for ruminants. As the plant matures, its dry matter (DM) content increases and its water soluble carbohydrates (WSCs) decrease as they are converted to starch (Weinberg *et al.*, 1991). These changes are associated with a relatively slow decline in the digestibility of organic matter. Reports have also indicated reduced protein and increased fiber levels, along with decreased neutral detergent fiber (NDF) digestibility (Weinberg *et al.*, 1991; Ariel and Adin, 1994), and increased pH (Wilkins *et al.*,

1970). Also, lactic acid and acetic acid contents fall (Acosta *et al.*, 1991) and ethanol and ammonia tend to decline (Tetlow, 1992) as DM or maturity at ensiling increases. Therefore, the feed value of the crop is a compromise between its DM yield and its digestibility (Holms, 1989). To improve the ensiling process, various chemical and biological additives have been developed (Kung *et al.*, 1987; Adesogan and Salawu, 2004; Kleinschmit and Kung, 2006). Several studies have shown the beneficial effects of bacterial inoculation on corn silage preservation (Johnson *et al.*, 2003; Rowghani and Zamiri, 2009). Microbial additives improved silage quality, nutrient digestibility and net energy for

lactation, and reduced protein degradation (Ilakova *et al.*, 1998; Filya, 2003a; Rowghani *et al.*, 2008). However, DM digestibility of treated silages was not affected in some studies (Rooke *et al.*, 1988; Kung *et al.*, 1993). With corn silage it is shown that the inoculants of lactic acid bacteria (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).

The objective of this study was to investigate the effects of a commercial bacterial inoculant applied at the time of ensiling on the fermentation characteristics, rumen degradability, and nutritive value of WPC. Harvesting time depends on the climate conditions and sometimes WPC can not be harvested at proper DM content. So another goal of the research was to find out if inoculation can improve the quality of WPC ensiled at three different dry matter contents.

Materials and Methods

Silage preparation

Based on local routine practice, WPC was harvested at three stages of maturity as follows: 1) two weeks before routine harvesting time, 2) one week before routine harvesting time, and 3) at routine harvesting time from a corn field of the College of Agriculture, Zabol University, Iran. A commercial inoculant (Ecosyl, Lallemand SA, SaintSimon, France) consisting of *Lactobacillus plantarum* was used. Two treatments were prepared for each harvesting time, 1: control (untreated), 2: WPC treated with inoculant at the rate of 1×10^5 colony forming units CFu/g of fresh forage. Bacterial counts were based on the manufacturer's recommendation. For preparation of each treatment, sufficient chopped (3-5 mm length) forage was placed on a polyethylene sheet and sprayed with the solutions of the inoculant, followed by thorough mixing. The same volume of water which was used to dissolve the additives was added to the control treatments to maintain equal moisture. Dark polyethylene bags were packed with 100 kg of each treated

forage. Two silo bags were used per treatment, kept indoors and opened after 25 days of ensiling. After 25 days, representative samples were taken from each treatment for chemical analysis and determination of rumen degradability, and *in vitro* gas production.

Chemical analysis

The dry matter (DM), ether extract (EE), organic matter (OM), ash and crude protein (CP) contents of silage samples were determined following the procedures of AOAC (2000). Neutral detergent fiber and acid detergent fiber (ADF) were measured according to the method of Goering and Van Soest (1970). The pH of each sample was determined in triplicate using 25 g of wet material added to 100 ml of distilled water. After homogenizing for 10 min in a blender, the pH was determined using a digital pH meter (Pye Unicam, Phillips). The water soluble carbohydrate (WSC) was determined by the method of Deriaz (1961). All analyses were carried out in triplicate.

In situ rumen degradability of DM

Rumen degradability was estimated *in sacco* (Orskov and McDonald, 1979). The dry samples from each treatment were ground (2-mm sieve), and approximately 5 g of each sample (DM) was transferred into polyester bags (12 × 19 cm) with 50- μ m pore size. Four bags per treatment and incubation time were incubated in the rumen of two ruminally fistulated Sistani bulls (450 kg BW) for 3, 6, 12, 24, 48 and 72 h. The cattle were fed a diet consisting of 80% of a mixture of wheat bran and alfalfa hay (50:50) and 20% corn silage (as fed basis) 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 washout. After each incubation time (including the zero h), the bags were removed and hand-washed with cold water until the water remained clear. Samples were then dried in an oven at 55°C until a constant weight was achieved before determination of DM disappearance. Loss of DM at various incubation intervals was fitted to the non-linear equation $p = a + b(1 - e^{-ct})$ (Orskov and

McDonald, 1979), in which

p: the amount degraded at time

a: the fraction that is soluble or immediately degraded

b: the fraction that is potentially degradable but insoluble

c: the fractional rate constant at which the fraction "b" will degrade per h

These data (a, b, c and p) were analysed by one way analysis of variance.

***In vitro* gas production (GP), *in vitro* organic matter digestibility (IVOMD), digestible organic matter in dry matter (DOMD), and metabolizable energy (ME) estimation**

In vitro incubation was performed using 30 ml of buffered rumen fluid according to the method of Menke and Steingass (1988). Approximately 200 mg of each sample was placed in 100 ml graduated glass syringes. The buffer mineral solution was prepared and placed in a water bath at 39°C under continuous flushing with CO₂. Rumen fluid was collected 2 h after morning feeding from two ruminally-fistulated Sistani bulls which were used in the rumen degradability experiment. Rumen fluid was pumped with a manually operated vacuum pump from the rumen into pre-warmed (39°C) thermos flasks. The rumen fluid from the two bulls were mixed and filtered through four layers of cheesecloth and flushed with CO₂. The thoroughly mixed and CO₂ flushed rumen fluid was added to the buffered mineral solution (1:2 v/v), which was maintained in a water bath at 39°C and mixed. Buffered rumen fluid (30 ml) was pipetted into each syringe. The syringes were immediately placed in a water bath maintained at 39°C. Gas production was recorded at 2, 4, 6, 8, 12, 24, 48, 72 and 96 h. To estimate the IVOMD and ME, triplicates of each sample were used and the GP was corrected for the GP of buffered rumen fluid with no sample. Estimated ME concentration and IVOMD of the samples were calculated as described by Makkar (2004).

Statistical analysis

The data were subjected to analysis of variance using General Linear Model procedure of SAS (1996). Mean separation

was performed by the Duncan's multiple range tests, and the level of significance was set at 5%.

Results

The chemical composition of corn silages treated and untreated with bacterial inoculant (Ecosyl) is shown in Table 1. There were significant effects (P<0.01) of harvesting time, inoculation and harvesting time × inoculation interactions on pH values. The consistent trend was that the pH values were lower for inoculated corn silages (except treatment 6) versus uninoculated corn silages. The pH of treatment 4 (3.50) was significantly (P<0.05) lower than treatments 1, 5 and 6 and treatment 3 was intermediate. There were harvesting time (P<0.05) and harvesting time × inoculation (P<0.01) interactions on DM content of the silages, and inoculated silages tended to have greater DM concentrations than uninoculated silages at each harvesting time. DM content of treatments 3 and 4 were significantly (P<0.05) lower than other silages. There were harvesting time (P<0.01), inoculation and harvesting time × inoculation (P<0.05) interactions for OM content. The OM content was significantly (P<0.05) higher for treatments 1, 3 and 4 than other treatments. There were significant effects of harvesting time (P<0.01), inoculation (P<0.05) and harvesting time × inoculation (P<0.01) interactions on ash content. Ash content was higher for treatments 5 and 6 and lower (P<0.05) for treatments 1, 3 and 4. There were harvesting time, inoculation and harvesting time × inoculation (P<0.05) interactions for silage WSC concentrations, and WSC concentrations were lower for inoculated silages compared with uninoculated silages. WSC content was significantly higher (P<0.05) for treatment 3 compared with other treatments. On 25 days of ensiling there were significant effects of harvesting time (P<0.05) and harvesting time × inoculation (P<0.01) interactions on CP content, and was higher for treatments 3 and 4. There were significant effects of harvesting time and inoculation (P<0.05) interactions on ADF and NDF contents and

treatment 4 had the lowest ($P<0.05$) ADF content. ADF content was significantly higher ($P<0.05$) for treatments 5 and 6. NDF content was significantly lower for treatment 3 and higher for treatment 6 ($P<0.05$). The significant harvesting time \times inoculation interactions for chemical composition of the silages reflect the differences in response to inoculation at different maturity levels. Inoculation and harvesting time were more effective on pH ($P<0.01$) values than other parameters.

The DM degradation kinetics (%) of silages are presented in Table 2. There were significant ($P<0.01$) effects of harvesting time, inoculation (except for "b" fraction) and harvesting time \times inoculation interactions on the "a", "b" and "a+b" fractions of DM degradation kinetics for all silages, but there were no significant interactions of harvesting time, inoculation and harvesting time \times inoculation on "c" fraction values for neither of the silages. There was only a significant ($P<0.01$) interaction for harvesting time on effective degradability (ED) of all silages. Fraction "a" was highest for treatment 4 compared with the other silages and was lowest for treatment 5 ($P<0.05$). Fraction "b" was highest for treatment 5 and lowest for treatment 4. The degradation rate (c) was highest for treatments 5 and 6 and lowest for treatment 1 ($P<0.05$). The maximum

potential degradability (a+b) was highest for treatments 1 and 2 and was lowest for treatment 4 ($P<0.05$). Effective degradability of DM was highest for treatment 4 and lowest for treatment 3 ($P<0.05$).

Table 3 presents *in vitro* GP, estimated IVOMD, DOMD and ME values of the silages. Except for 4 and 96 h. of incubation, there was no significant interaction of inoculation on GP, but there were significant ($P<0.01$) interactions of harvesting time and harvesting time \times inoculation for all incubation h, which is largely due to the differences in response to maturity levels of different harvesting times. From 2 h of incubation, the GP was higher for treatments 2, 3, 4 and 5 and lower for treatment 1 ($P<0.05$). Between 4 and 8 h, GP had the same trend and was higher for treatment 4 and lower ($P<0.05$) for treatment 6 (except for 6 h). From 12 up to 24 h, GP was higher ($P<0.05$) for treatment 4 than other treatments but from 48 h up to 96 h, GP was higher for treatments 4 and 5 and lower ($P<0.05$) for treatment 1.

There were harvesting time ($P<0.01$), inoculation and harvesting time \times inoculation interactions ($P<0.01$) on IVOMD, DOMD and ME contents of silages and treatment 4 had the highest values compared with other treatments. Again, the significant harvesting time \times inoculation interaction reflects the differences in

Table 1: The chemical composition (DM basis) and pH of corn silages after 25 days of ensiling

Parameters	Harvesting time [†]						SEM	Interaction ^{†††}		
	1		2		3			H	I	H×I
	Treatments ^{††}									
	1	2	3	4	5	6				
pH	3.71 ^a	3.52 ^c	3.62 ^b	3.50 ^c	3.73 ^a	3.54 ^a	0.001	**	**	**
DM	19.77 ^c	21.60 ^b	18.63 ^d	19.05 ^{cd}	24.21 ^a	24.37 ^a	0.231	*	NS	**
OM	83.32 ^a	81.69 ^b	83.83 ^a	83.71 ^a	78.65 ^c	78.80 ^c	0.246	**	*	*
Ash	16.67 ^c	18.30 ^b	16.16 ^c	16.28 ^c	21.34 ^a	21.19 ^a	0.246	**	*	**
WSC	2.18 ^{bc}	2.15 ^{bc}	4.46 ^a	2.47 ^{bc}	2.71 ^b	1.42 ^c	0.381	*	*	*
CP	9.08 ^c	8.79 ^d	10.24 ^a	10.29 ^a	8.35 ^d	9.32 ^b	0.012	*	NS	**
ADF	40.48 ^b	40.02 ^b	39.34 ^b	37.39 ^c	43.29 ^a	42.15 ^a	0.853	*	*	NS
NDF	61.18 ^d	63.28 ^c	56.62 ^f	58.05 ^e	64.72 ^b	66.53 ^a	0.106	*	*	NS

†: 1, 2 and 3 are three harvesting times, respectively. ††: treatments 1 and 2: untreated and inoculant-treated whole-plant corn ensiled two weeks before routine harvesting time, respectively, treatments 3 and 4: untreated and inoculant-treated whole-plant corn ensiled one week before routine harvesting time, respectively, treatments 5 and 6: untreated and inoculant-treated whole-plant corn ensiled at routine harvesting time, respectively. †††: H = Harvesting time, I = Inoculation, HI = Harvesting time \times Inoculation. Means within a row with similar superscript(s) are not significantly different (Duncan's test, $P>0.05$). NS = Not significant ($P>0.05$). *: $P<0.05$ and **: $P<0.01$

Table 2: Dry matter degradation kinetics (%) of corn silages

Constants	Harvesting time [†]						SEM	Interaction ^{†††}		
	1		2		3			H	I	H×I
	Treatments ^{††}									
1	2	3	4	5	6					
“a”	23.21 ^d	31.03 ^b	28.90 ^c	36.06 ^a	18.39 ^f	21.01 ^e	1.21	**	**	**
“b”	47.46 ^{ab}	39.13 ^c	40.17 ^c	37.06 ^d	48.69 ^a	46.48 ^b	1.25	**	NS	**
a+b	70.67 ^a	70.17 ^a	69.09 ^b	63.13 ^d	67.08 ^c	67.50 ^c	0.376	**	**	**
“C” (h ⁻¹)	0.046 ^c	0.059 ^b	0.051 ^{bc}	0.058 ^b	0.093 ^a	0.096 ^a	0.0003	*NS	NS	NS
ED	51.52 ^c	52.26 ^b	49.20 ^e	56.00 ^a	50.10 ^d	51.60 ^c	0.08	**	NS	NS

†, †† and †††: see Table 1 for details. a+b: Potential degradability. Degradability values calculated at 0.05 per hour outflow rate. Means within a row with similar superscript(s) are not significantly different (Duncan's test, P>0.05). NS = Not significant (P>0.05). *: P<0.05 and **: P<0.01

Table 3: GP (ml g⁻¹ DM) at different hours of incubation and estimated ME (MJ kg⁻¹ DM), IVOMD (g/kg) and DOMD (g kg⁻¹) of corn silages

Gas production after (h)	Harvesting time [†]						SEM	Interaction ^{†††}		
	1		2		3			H	I	H×I
	Treatments ^{††}									
	1	2	3	4	5	6				
2	10.83 ^b	13.31 ^a	13.61 ^a	13.88 ^a	13.36 ^a	11.76 ^{ab}	1.36	*	NS	**
4	19.94 ^b	20.05 ^b	20.27 ^b	21.13 ^a	13.20 ^b	18.31 ^c	0.08	**	*	**
6	28.38 ^b	28.71 ^b	28.38 ^b	29.84 ^a	28.15 ^b	25.76 ^a	0.11	**	NS	**
8	34.21 ^b	34.57 ^b	34.70 ^b	36.62 ^a	34.04 ^b	31.93 ^c	0.12	**	NS	**
12	44.55 ^{cd}	44.97 ^c	46.54 ^b	48.87 ^a	46.49 ^b	44.11 ^d	0.14	**	NS	**
24	56.71 ^f	57.54 ^e	61.85 ^c	64.74 ^a	62.78 ^b	60.10 ^d	0.19	**	NS	**
48	66.60 ^d	68.15 ^c	72.39 ^b	75.55 ^a	74.60 ^a	71.49 ^b	0.46	**	NS	**
72	68.26 ^d	70.48 ^c	71.74 ^b	77.53 ^a	77.40 ^a	77.14 ^b	0.55	**	NS	**
96	71.21 ^d	73.68 ^c	77.56 ^b	81.10 ^a	80.65 ^a	77.33 ^b	0.64	**	*	**
ME	10.65 ^f	10.81 ^e	11.48 ^b	11.90 ^a	11.02 ^d	11.33 ^c	0.003	**	*	**
IVOMD	80.27 ^e	82.25 ^d	85.03 ^c	87.78 ^a	85.84 ^b	87.07 ^a	0.15	**	**	**
DOMD	66.89 ^e	67.19 ^{de}	71.29 ^b	73.48 ^a	69.27 ^c	67.64 ^d	0.10	**	*	**

†, †† and †††: see Table 1 for details. DOMD calculated as: DOMD = IVOMD × %OM. Means within a row with similar superscript(s) are not significantly different (Duncan's test, P>0.05). NS = Not significant (P>0.05). *: P<0.05 and **: P<0.01

response to the inoculation at different stages of maturity of WPC. The IVOMD was significantly higher for treatments 4 and 6 and lower for treatment 1 (P<0.05). The DOMD was highest for treatment 4 and lowest for treatment 1 (P<0.05). The ME value was highest for treatment 4 and lowest for treatment 1 (P<0.05).

Discussion

The stage of harvesting has a great effect on DM content and chemical composition of forages (Di Marco *et al.*, 2002). The higher DM content of treatments 5 and 6 is related to the higher stage of harvesting (Jones *et al.*, 1992), while the lower DM content of silages 3 and 4 might be due to more extensive fermentation (McDonald *et al.*, 1991). All silages had pH values less than

4.0, indicating successful preservation and fermentation. The lowest final pH value of treatment 4 (3.50) can potentially minimize the growth of Clostridia due to the low concentration of butyric acid (McDonald, 1981). The OM content of treatment 4 was higher than treatments 2 and 6 which may be the result of more effectiveness of the addition of bacterial inoculant to WPC harvested at one week before routine time. This finding, however, is not in agreement with the findings of Meeske *et al.* (2002) who reported no increase in the OM content of inoculant-treated big bale oat silage compared with the control silage. The higher ash content of treatments 5 and 6 might be due to the higher DM content and harvesting time which is not in agreement with the report of Fisun Koc and Ozdven (2008), who reported a decrease in ash content of

inoculant-treated sunflower silages. These differences in the results may be due to the differences in ensiled materials. Residual WSC content of the forages decreased with advancing maturity (Jones *et al.*, 1992; Filya, 2004) and the higher WSC content of treatment 3 might be due to the more acid hydrolysis of cell wall carbohydrates during ensiling (McDonald *et al.*, 1991). Lower WSC content of the inoculated silages may be a result of the higher microbial population and fermentation activity (Hassanat and Mustafa, 2007). The CP content decreased with advancing maturity (Filya, 2003b; Başkavak *et al.*, 2008). Higher CP content of the treatment 4 could be from the addition of microbial inoculant and less proteolytic activity (Chamberlain *et al.*, 1990; Rooke *et al.*, 1988) in the silage, which is in agreement with the findings of Mansoori *et al.* (2008). Lower ADF content of treatment 4 is an indication of higher cell wall digestion during fermentation (Bolsen *et al.*, 1996). Lower NDF content of treatments 3 and 4 might be due to the acid hydrolysis of fiber (McDonald, 1981) as a result of more fiber degradation at this DM content of WPC and more microbial activity. Fraction "a" of DM degradation kinetics was higher but fraction "b" was lower ($P < 0.05$) for treatment 4, reflecting the inverse relationship between these fractions which could be due to the effect of microbial inoculation in degrading the lignocellulose fraction of the cell wall and providing more soluble materials for the rumen microbes (Bolsen *et al.*, 1996; Adesogan and Salawu, 2004). The potential (a+b) DM degradability was higher ($P < 0.05$) for treatments 1 and 2, possibly because of the synergistic effects of more digestibility of younger WPC and the effect of microbial inoculant compared to other treatments. ED was higher for treatment 4 reflecting the probable ability of the microbial inoculant in enhancing rumen degradability. Hunt *et al.* (1989) and Russell *et al.* (1992) found that despite declines in NDF and ADF contents, *in situ* rumen degradability decreased progressively with advancing maturity of corn silage, which is not in agreement with the findings for treatment 4 in the present study, which again may be due to the beneficial effect of microbial inoculant in enhancing the

nutritive value of corn silage. From 4 h up to 24 h of incubation, the GP was significantly higher ($P < 0.05$) for treatment 4 than for other silages and from 48 up to 96 h of incubation, the GP was higher ($P < 0.05$) for treatments 4 and 5. GP has a negative relation with NDF content and a positive relation with starch content of the material (De Boever *et al.*, 2005). Also, it has been reported that GP in phase 1 (fermentation of soluble fraction) of incubation was affected by soluble fraction "a" for corn silage (Cone *et al.*, 1997). These findings are in agreement with treatment 4 in the present study which had higher GP, lower NDF content and higher fraction "a". GP was positively correlated to IVOMD, DOMD and ME, which is in agreement with the data of Al-Masri (2003). Van Soest and Robertson (1985) showed a highly significant and positive relationship between GP and the *in vitro* apparent and true degradabilities. Treatment 4 had higher ($P < 0.05$) IVOMD, DOMD and ME values than other silages which may show the beneficial effect of adding microbial inoculant to WPC harvested at one week before routine harvesting time and hence, having a higher nutritive value. These findings are in agreement with the previous reports (Ilakova *et al.*, 1998; Aksu *et al.*, 2006). Higher nutrient digestibility has been reported with microbial inoculant-treated corn silage (Anderson *et al.*, 1989; Kung *et al.*, 1993). Flexibility of harvesting and ensiling of WPC at different stages of maturity has advantages in unstable weather, especially when double cropping is being practiced and time is limited.

Based on the results of the present study, the addition of microbial inoculant containing *L. plantarum* to WPC ensiled at one week before routine harvesting time, produced well-fermented silages with higher effective degradability, higher IVOMD, DOMD and ME and guarantee a well-preserved and high nutritive value feedstuff for ruminants. Additional farm trials, however, are needed to confirm the findings of the present study.

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