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Research Article

Effect of Intercropped Corn and Soybean Silage on Nutritive Values, *in vitro* Ruminal Fermentation, and Milk Production of Holstein Dairy Cows

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ABSTRACT

This study was conducted to examine the effect of corn (*Zea mays* L.) - soybean (*Glycine max* L.) silage prepared by intercropping method on the nutritive value of the silage, *in vitro* rumen fermentation characteristics, dry matter degradability, as well as milk yield and milk composition of dairy cows. In a couple of experiments intercropped corn-soybean silage (CSBS) was compared with corn silage (CS) and/or Italian ryegrass hay (IRG). Numerically, CSBS had higher crude protein, ether extract, and lactic acid contents compared to CS. *In vitro* rumen fermentation analysis demonstrated that up to a 24-h incubation period, both CS and CSBS showed higher total gas production, ammonia N concentration, and dry matter degradability compared to IRG (p<0.05). The investigation on animals was conducted in a commercial dairy farm located in Gyeongju, South Korea, employing 42 Holstein cows that were divided into 2 group treatments: CS and CSBS in a completely randomized design. Although no significant difference was observed in milk yield, animals fed on CSBS showed significantly higher milk protein (p<0.05) and milk fat content (p<0.01), compared to animals fed on CS. Taken together, our findings indicate that corn-soybean silage that is cultivated, harvested, and prepared through intercropping can improve the protein content of the silage, and can also enhance *in vitro* rumen fermentation, dry matter degradability, and performance of dairy cattle.

(**Key words** : Intercropping, Corn, Soybean, Milk production, Dairy cow)

Ⅰ. INTRODUCTION

For ruminant production, including Hanwoo beef and Holstein dairy cows, good quality forages play an important role by providing animals with fundamental nutrients for their physiological activities as well as meat and milk production. Recently, forage-based feeding has become increasingly important in response to the consumer's demand for animal welfare and products from animal-friendly farms.

With recent efforts by the central government (see http://english.mafra.go.kr/main.jsp), forage production has increased rapidly in terms of quality and quantity. Nevertheless, the amount of good quality forage produced in Korea, excluding rice straw, is approximately 2 million tons and this satisfies only 42% of the domestic requirement in Korea (Yang et al., 2016). Therefore, it is important for agricultural scientists in Korea to research how to increase forage production and its nutritive values for animals.

Corn, as a forage crop, has been studied for many decades worldwide. Forage corn is a crop with several benefits, including high in dry matter yield per unit land area and superior palatability by ruminants. Recently, forage corn and its grain has been used for recycling energy (i.e., bioethanol) in the United States and other countries (Klopfenstein et al., 2008; Park et al., 2016; Son et al., 2014). In Korea, forage corn is generally produced in the form of silage, and supplied to ruminants as part of a total mixed ration (TMR) or as a supplemental forage to other available forage sources. Although corn has many advantages as a forage crop, it has lower crude protein content than other home-grown or imported grass and leguminous forages. Additionally, its amino acid profile is not considered adequate for ruminants, and as such protein supplements are often required when forage corn is supplied to ruminants (Cabrita et al., 2011; Felix et al., 2014). As a way of overcoming the crude protein deficiency of forage corn, some studies have mixed corn with legume forages (Baxter et al., 1984; Budakli, 2016; Yang et al., 2016) whereas others produced silages from corn-soybean intercropping (Martin et al., 1990). Specifically,

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a method of mixed-cropping corn with a legume forage improved dry matter and crude protein yield, and subsequently animal performance (Esmail, 1991; Martin et al., 1990). Limited efforts have been attempted in Korea (Yang et al., 2016). However, this research was mainly focused on forage production, and information on animal performance was never reported. Therefore, this study was conducted to examine the effects of the corn-soybean silage that was prepared by an intercropping method on nutritive values, *in vitro* ruminal fermentation characteristics, and milk production and composition in Holstein dairy cow.

Ⅱ. MATERIAL AND METHODS

1. Analysis of experimental silages

The corn silage (CS) and corn-soybean mixed silage (CSBS) were commercially cultivated, harvested, and prepared (approximately 500 kg/bale) in 2015 at the cultivation area (36.016951, 129.233703) located in Gyeongju, Korea, using either corn (*Zea mays*, c.v. Pioneer-35P75) or corn and soybean (*Glycine max* c.v. P-I483463×Hutcheson). Although silage making was not part of this study and we have purchased CS and CSBS once they were prepared at the commercial farm described above, brief details were available. Intercropped corn-soybean silage was seeded with ratio of 1:3-4 (corn:soybean, 15 kg/ha or corn and 15 kg/ha of soybean) on either 5 or 15 June 2015 and, harvested between 23 September and 8 October 2015. Silages were immediately prepared as a bale (approximately 500 kg weight as fed basis) without wilting and kept on the ground for at least 40 days prior to chemical analysis, *in vitro* and dairy cow study (see below). Corn silage was prepared at the same time and silage additives were not used for both silages. Fertilizer applied for both corn and corn-soybean intercropping was the mixture of N, P, K with the ratio of 21:17:17 (1 tonne/ha).

These silages were used in the following *in vitro* study and dairy cattle experiment. One percent (approximately 5 kg) of each representative silage was manually sampled to analyze the chemical composition. The silages were brought to the laboratory and immediately dried in a 65°C drying oven for 72 h. After being weighed, the dried samples were ground with a high-speed grinder (ZM200, Retch, Germany) with a 1 mm mesh for use in proximate analyses (Table 1). In addition, 100 g of fresh silage was mixed with 900 mL of distilled water and homogenized using Stomacher (Bag Mixer, Interscience Inc., Korea; speed level 4) for 4 min. The supernatant was filtered and the pH immediately measured. The supernatant was placed in 2 mL microcentrifuge tubes and stored at -20°C for the analysis of ammonia nitrogen (N), lactic, and volatile fatty acids (VFA).

2. Evaluation of *in vitro* ruminal fermentation characteristics

For this study, 24-month-old Hanwoo cows (n=3) were maintained at the experimental farm in Sangju, Kyungpook National University. The Hanwoo cows were fed twice a day (08:00 in the morning and 17:00 in the evening) with 5 kg of concentrate and *ad libitum* rice straw. Rumen fluid was collected from each cow 2 h after the morning feeding using a stomach tube. Samples were immediately stored in a 2 L thermos and transported to the laboratory with no headspace to minimize oxygen penetration in the thermos bottle. The collected rumen fluid was double filtered with 4 layers of medical gauze. Diluted rumen fluid was prepared by mixing the filtered rumen fluid with McDougall's artificial saliva McDougall (1948) at a ratio of 1:4. In addition, the rumen fluid was maintained in anaerobic conditions with continuous bubbling by CO_2 gas. Aliquots of 0.3 g ground samples and 0.2 g concentrate feed (at a ratio of 3:2) were weighed precisely and added to a 125 mL serum bottle. Italian ryegrass (*Lolium multiflorum*), which is frequently used in the field, was added as a control. The chemical composition of the experimental diets is presented in Table 1.

The incubation procedure was carried out according to the method of Tilley and Terry (1963). Forty mL of diluted rumen fluid were injected into the serum bottle containing the sample, sealed with a butyl rubber stopper and aluminum cap, and incubated for 3, 6, 12, and 24 h in an incubator set at 39°C. At the end of each incubation period, the amount of gas generated was measured and the culture solution was collected. The content of serum bottles was transferred to nylon bags (50 μm pore, 5 cm × 10 cm, ANKOM, USA) to separate the culture medium from the remaining substrates. The pH of the collected culture medium was immediately measured (pH meter, Starter 2100, Ohaus, USA) and the medium was centrifuged (Labogene, 1730MR, Korea) at 4°C for approximately 10 min at 10,000 rpm. The supernatant was stored at -20°C for ammonia-N and VFA analyses. The nylon bag was washed with tap water and distilled water until the water ran clear and then dried at 105°C in a drying oven (Wiseven, Nof-155, Korea) for 24 h, to determine dry matter degradability. At zero-time incubation, the degradability and fermentation characteristic were measured directly after the inoculation of rumen fluid in the same manner as the other incubation periods.

3. *In vivo* dairy cow study

The *in vivo* dairy cow study was conducted in a dairy farm (36.008872, 129.203680) located in Angang, Gyeongju, to investigate the effects of corn-soybean intercropped silage on milk production. Forty-two Holstein cows were assigned as experimental animals (average number of parity: 2.5, average milking period: 198 days) in a completely randomized design. This study was conducted for 9 weeks. The experimental feed consisted of corn silage (CS) and corn-soybean mixed silages (CSBS), with 10 kg provided daily as part of a total mixed ration (TMR). Feed was offered twice a day after milking at 07:00 and 18:00. Fresh water and mineral blocks were given *ad libitum*.

Forty-two cows were assigned the CS or CSBS treatments with 24 and 18 cows, respectively. During the study, three cows from the CS treatment were excluded due to illness and initiation of a dry period. CS and CSBS were gradually introduced to the cows for 7 weeks with the basic feed left unchanged. From weeks 7 to 9, 10 kg of the experimental silage, 10.9 kg of the TMR, and 3 kg of commercial dairy cow feed were provided in the same manner. The nutrient values of the silages used in this study are shown in Table 1. The TMR nutrient values are obtained from the feed manufacturer in Gyeongju. The TMR consisted of 35% formulated feed, 5% water, 11.7% alfalfa hay, 2% Italian ryegrass, 7% oat, 5% timothy, 5% probiotics, 10% whole cotton seed, 3% beet pulp, 10.5% crushed corn, 4% molasses, 1.5% limestone, and 0.3% protected fat. Feed intake for each individual cow was not determined as they were not individually penned. Milk samples were collected at 07:00 and 18:00 at the end of the experiment for 3 days. The amount of milk production was calculated by summing the morning and afternoon milk production. Milk samples were stored in an ice box and transferred to the laboratory on the same day. Milkoscan (CA3-45, Delta, Netherlands) was used to analyze the milk fat, milk protein, lactose concentration, solid not fat (SNF), milk urea N (MUN), and somatic cell count (SCC).

Table 1. Nutritive values of corn silage and intercropped corn-soybean silage, and the chemical composition of the experimental diets used for *in vitro* experiments (% of dry matter unless otherwise stated)

Item	CS[1]	CSBS[2]	Concentrates	Italian ryegrass
Fermentation characteristics				
pH	3.92	3.99	-	-
Ammonia N (mg/100 mL)	2.62	2.48	-	-
Lactic acid (g/kg DM)	47.56	58.46	-	-
Acetic acid (g/kg DM)	6.78	7.61	-	-
Chemical composition				
Dry matter (as fed)	39.59	35.61	84.10	86.87
Organic matter	94.93	95.75	94.45	95.13
Crude protein	8.34	8.97	17.34	4.06
Ether extract	4.24	4.55	6.66	0.91
Neutral detergent fiber	36.72	37.10	28.59	79.30
Acid detergent fiber	17.25	17.82	14.99	52.96

[1] CS = corn silage

[2] CSBS = intercropped corn-soybean silage

4. Chemical analysis

Dry matter (DM), organic matter (OM), crude protein (CP), and ether extract (EE) were analyzed according to the AOAC method (1995), whereas neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed according to the Goering and Van Soest method (1970). Neutral detergent fiber analysis was performed using sodium sulfite and α-amylase. The ammonia concentrations in the silage and *in vitro* fermentation cultures were determined by using the Chaney and Marbach method (1962), whereas the VFA concentration was determined according to Erwin et al. (1961). In order to analyze the VFA concentration, 1 mL of filtered liquid from *in vitro* cultures was mixed with 0.2 mL of 25% metaphosphoric acid (Wako, Japan) and centrifuged at 10,000 rpm for 10 min (Labogene, 1730MR, Korea). The supernatant was collected and analyzed using gas chromatography (Bruker Inc., 450-GC, Germany). The lactic acid concentrations in the silages were measured by high performance liquid chromatography (Perkin Elmer, Series 200) equipped with Aminex® HPX-87H (300 mm × 7 mm) after filtering the stomaching process supernatant through a 0.45 μm syringe filter.

5. Statistical analysis

The effects of corn and corn-soybean intercropped silages on *in vitro* rumen fermentation characteristics and degradability were analyzed using one-way analysis of variance (ANOVA). The effect of incubation time was not examined. Significance was tested using Duncan's multiple range test at a significance level of 5%. Student's *t*-test was performed on the animal experiments results. The statistical analysis was performed using SPSS (IBM Corporation, 2013).

Ⅲ. RESULTS AND DISCUSSION

1. Chemical composition of corn-soybean intercropped silage

The pH of the intercropping corn-soybean silage was similar to that of corn silage, at 3.9. The concentration of acetic and lactic acids were also comparable with that in corn silage, despite the inclusion of legume forage in the silage (Table 1). Due to the limited literature available, especially from studies in Korea, it is difficult to compare our results with published data. Lee and Park (2002) demonstrated the importance of legume forages for the production of good quality forages suitable for organic animal production and demonstrated the additional benefit of nitrogen fixation by the legume forage. Lee et al. (1997) reported the growth characteristics and dry matter yield when sorghum × sudangrass hybrids were intercropped with soybean. Recently, grass species such as whole-crop wheat and legume forage intercropping has also been reported in terms of nutritive yields and feed values for ruminants (Hwangbo and Jo, 2014). In terms of silage pH, all treatments have shown a value below 4.0, suggesting that the fermentation process during ensiling was stable and that the concentration of lactic and acetic acids were in a range indicating good quality silage (Phelan et al., 2015). Our results on chemical composition are in agreement with those of Yang et al. (2016) who demonstrated effects of corn and wild-type soybean mixed silage. They are also consistent with the results of other studies that reported nutritive values of legume forage mixed silages (Erdal et al., 2016; Htet et al., 2016). For legume forages, it is recognized that making silage is more difficult than grass species because of relatively higher crude protein content and buffering capacity. This prevents the rapid drop of pH at the early stages of fermentation and provides a potential environment for unfavorable microorganisms to grow (Phelan et al., 2015). However, in the case of silages with intercropping between corn and soybean, higher levels of water-soluble carbohydrates and starch in corn rapidly lowers the pH at early stages of fermentation and maintains stable fermentation conditions throughout the ensiling process (Phelan et al., 2015).

2. Ruminal fermentation characteristics and dry matter degradability *in vitro*

The results from the *in vitro* ruminal fermentation characteristics and dry matter degradability are presented in Table 2. For a better comparison, Italian ryegrass hay (IRG)

Table 2. Effects of intercropped corn-soybean silage on *in vitro* ruminal fermentation characteristics

Incubation time (h)	Treatments			SEM[4]	Significance
	IRG[1]	CS[2]	CSBS[3]		
pH					
3	6.79	6.80	6.80	0.008	NS[5]
6	6.75	6.76	6.76	0.008	NS
12	6.76	6.73	6.74	0.010	NS
24	6.77[b]	6.68[a]	6.64[a]	0.021	*
Total gas production, mL					
3	8.00	8.17	8.67	0.345	NS
6	16.00	15.67	15.50	0.345	NS
12	25.17[a]	33.83[b]	34.50[b]	1.521	*
24	47.83[a]	59.33[b]	62.67[c]	2.262	*
Ammonia N, mg/100 mL					
3	1.32[a]	1.90[b]	1.68[b]	0.104	*
6	0.88[a]	1.37[b]	1.32[b]	0.080	*
12	0.81	0.83	0.85	0.014	NS
24	0.98	0.90	0.96	0.021	NS
Dry matter digestibility (%)					
3	28.07[a]	39.03[c]	36.00[b]	1.686	*
6	26.19[a]	39.80[b]	37.28[b]	2.157	*
12	28.73[a]	45.88[c]	43.52[b]	2.695	*
24	36.91[a]	56.24[b]	54.79[b]	3.139	*
Total VFA[6] (mM)					
3	21.47	21.39	22.72	0.390	NS
6	28.93	26.40	28.21	0.474	NS
12	36.88	40.44	39.86	0.735	NS
24	49.37[a]	63.43[b]	66.37[b]	2.725	*
Acetic acid (% of total VFA)					
3	75.72[a]	76.72[b]	77.29[b]	0.270	*
6	75.98[b]	74.55[a]	75.53[b]	0.229	*
12	70.42[b]	68.60[a]	69.18[a]	0.294	*
24	65.03[b]	59.69[a]	59.74[a]	0.890	*
Propionic acid (% of total VFA)					
3	12.38[a]	13.08[c]	12.73[b]	0.108	*
6	13.40[a]	14.50[c]	14.04[b]	0.166	*
12	18.52[a]	20.78[b]	20.05[b]	0.395	*
24	24.54[a]	30.52[b]	31.04[b]	1.049	*
Butyric acid (% of total VFA)					
3	9.48	9.28	9.09	0.073	NS
6	9.40[ab]	9.57[b]	9.29[a]	0.051	*
12	9.75	9.18	8.55	0.250	NS
24	8.88[c]	8.31[b]	7.87[a]	0.156	*

[1] IRG = Italian ryegrass
[2] CS = corn silage
[3] CSBS = intercropped corn-soybean silage;
[4] SEM = standard error of the mean
[5] NS = not significant, *= $p<0.05$
[6] VFA = volatile fatty acids
[a, b, c] Means with different superscripts in the same row differ significantly ($p<0.05$).

was included in the experimental design due to its heavy use in the ruminant industry (and in the experimental farms as well). The ratio of forage:concentrate (60:40) did not cause any detrimental effects on pH, such that the pH was around 6.5 even at 24 h incubation time in all treatments. In terms of total gas production, ammonia concentration, and dry matter degradability, both CS and CSBS were higher ($p<0.05$) than IRG at most incubation periods. This suggests that CS and CSBS have greater utilization by ruminal microbes than IRG. However, the difference between CS and CSBS was relatively marginal. In the study by Yang et al. (2016), intercropped silage between corn and wild-type soybean have around 51-52% of *in vitro* dry matter degradability. In this study, we hypothesized that the increased concentration of crude protein content in CSBS would contribute to better ruminal fermentation. However, the difference in terms of ruminal fermentation *in vitro* appeared to be marginal. Therefore, further agronomic studies are needed to examine the crude protein content of CSBS. Total VFA concentrations were highest in CSBS ($p<0.05$) with a value of 66.37 mM and lowest in IRG (49.37 mM). In particular, propionate concentrations were significantly different among treatments. As evidenced by several reports, this may be attributable to the content of non-structural carbohydrates (i.e., starch) in CSBS which contribute to higher propionate and lower acetate production in the rumen (McDonald et al., 1995).

3. Effect on milk production and composition

Milk yield did not differ between CS and CSBS. However, milk fat ($p<0.01$) and milk protein content ($p<0.05$) were significantly higher in animals offered CSBS compared with those of CS (Table 3). Milk urea N had approximately 10.2 mg/dL for both CS and CSBS and free fatty acids were not different among treatments (Table 3). Somatic cell count was numerically lower in CSBS, yet this difference was not significant. Notably, the relatively marginal difference in terms of crude protein content between CS and CSBS contributed to differences in milk production. Presumably, this is due to a greater supply of crude protein and an improved amino acid profile within CSBS. As discussed in the introduction, corn silage has limitations in terms of crude protein content and its amino acid profile. Therefore, inclusion of soybean silage may have contributed to the amino acid profile within the rumen. Indeed, in the study by Esmall (1991), the concentration of lysine and methionine within corn-soybean silage increased compared with those of corn silage only. Due to its nutritive values and animal performance, the use of legume silage with corn silage have been reported elsewhere elsewhere (Felix et al., 2014; Moorby et al., 2016). In summary, our results demonstrate that corn-soybean silage prepared by intercropping improves nutritional value of the silage, ruminal fermentation, dry matter degradability, and provides better performance in dairy cows.

Table 3. Effects of intercropped corn-soybean silage on milk yield and milk composition, somatic cell counts of Holstein dairy cows

	CS (n = 15)[1]	CSBS (n = 24)[2]	SEM[3]	Significance
Milk yield (kg/d)	32.1	30.7	2.63	NS[4]
Fat (%)	3.85[b]	4.53[a]	0.200	**
Protein (%)	3.13[b]	3.36[a]	0.110	*
Lactose (%)	4.71	4.78	0.086	NS
Solid not fat (%)	8.56[b]	8.85[a]	0.111	*
Milk urea N (mg/dL)	10.28	10.19	0.547	NS
Free fatty acids (mg/mL)	0.48	0.55	0.111	NS
Somatic cell counts (×1000)	595	209	206.6	NS

[1] CS = corn silage
[2] CSBS = intercropped corn-soybean silage
[3] SEM = standard error of the mean
[4] NS = not significant, *= $p<0.05$, ** = $p<0.01$
[a, b, c] Means with different superscripts in the same row differ significantly ($p<0.05$).

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