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Comparative evaluation of nutritional values in different forage sources using *in vitro* and *in vivo* rumen fermentation in Hanwoo cattle

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Abstract

This study evaluated the nutritional value of Italian ryegrass (IRG) as a forage source for Hanwoo. The nutritional value of IRG was assessed and compared to that of rice straw, oat hay, and timothy hay using two different methods: 1) *in vitro* ruminal fermentation 2) *in vivo* total tract digestibility. *In vitro* DM digestibility was lower in rice straw compared to the other three forage sources after both 24 and 48 h of incubation ($p < 0.01$). Among the four forage sources, IRG had a higher NH_3-N concentration after both 24 and 48 h of incubation ($p < 0.01$). In the *in vivo* digestibility trials, four different substrates were used: 1) 80% concentrate with 20% rice straw, 2) 80% concentrate with 20% oat hay, 3) 80% concentrate with 20% IRG, and 4) 80% concentrate with 20% timothy hay. The dry matter, crude protein, non-fiber carbohydrate, and detergent fiber digestibility were the greatest in the C80-IRG20 among the four forage groups. In summary, IRG had a similar level of energy efficiency compared to oat hay and timothy hay. Furthermore, the result of the chemical composition analysis showing a higher ammonia concentration in the *in vitro* fermentation experiment and the high protein digestibility in the *in vivo* experiment indicate that IRG is a good source of protein compared to oat hay and timothy hay.

Keywords: feed-value evaluation, forage, Hanwoo, *In vitro* rumen fermentation, *In vivo* digestibility

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Introduction

The livestock industry in Korea, which relies mostly on imported feed resources, is facing many problems. These include increases in production costs arising from rapidly changing international grain prices and logistics costs. Research on domestic forage is also important for the health of ruminants. Forage is an important factor in the development of the rumen and the healthy growth of ruminants (Cho et al., 1997).

High quality forage involves a high concentration feed to forage ratio and is needed to maintain the physiology of Hanwoo cattle (Hanwoo) ruminants. Hanwoo farmers still primarily use concentrate feed, with rice straw as the forage. However, rice straw is a low-quality feed that may lack some specific nutrients (Seo et al., 2005).

Oat hay and timothy hay are commonly used imported forages in Korea. Farmers have a tendency to believe that domestic forages are not as good as those that are imported, which is not always the case (Lee and Lee, 2000). Moreover, good quality domestic forages can benefit Hanwoo production in ways that imported forages cannot, such as reducing production costs and adverse environmental impacts (Sung et al., 2010).

Interest in domestic forages such as Italian ryegrass, whole-crop barely, whole-crop rice, and oat hay has increased in recent years. Italian ryegrass could be grown domestically and could replace imported forages. It grows well in wet ground, such as drained paddy fields in winter, and is widely used as a second crop after the summer harvest, making it very suitable for growth in Korea (Kim et al., 1998; Kim and Cho, 2018). Italian ryegrass is used in a variety of ways─e.g., as hay, silage, and grazing─because of the total volume of dry matter (DM) production per area and its high total digestible nutrients (Kim et al., 2001). In addition, Italian ryegrass has good palatability and its high sugar content (15.15% DM) makes it suitable for high-quality silage production. A previous comparison between Italian ryegrass and rice straw found that the former has a higher average daily growth rate, Rate of appearance 1 quality grade in beef, and marbling score (Kim et al., 2018).

Although the TDN, crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) of IRG have been analyzed, there is still a lack of information regarding the feed values of domestic IRG in Korea. Furthermore, one of the problems of using IRG is that there is no nutritional information on domestic IRG forages.

Evaluating the nutritional value of feed resources is very important for both animal nutrition and economic considerations. However, to the best of our knowledge, no study has collectively examined the efficacy of IRG, rice straw, oat hay, and timothy hay on feed quality, ruminal fermentation, and digestibility in Hanwoo. In this study, a comparative analysis of the energetic value of IRG was conducted *in vitro* and *in vivo* along with the chemical analyses of three commonly used forages (rice straw, oat hay, and timothy hay) in Korea.

Materials and Methods

The protocols used for the animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the National Institute of Animal Science (No. 2018-301).

Chemical analysis

IRG, rice straw, oat hay, and timothy hay were used for chemical analysis. Before nutrient analysis, all the samples were dried at 60℃ for 48 h and ground in a cyclone mill (Foss, Hillerød, Denmark) fitted with a 1-mm screen. Dry matter (DM; 930.15), acid detergent fiber (ADF; 973.18), ash (942.05), and ether extract (EE; 2003.05) was determined as described by the AOAC (2016). Neutral detergent fiber was analyzed using a heat-stable amylase and was expressed inclusive of residual ash (aNDF) as described by Van Soest (1991). Crude protein was calculated as 6.25 times nitrogen content, and total nitrogen was measured using the Dumas combustion method, using an elemental combustor (Vario Max Cube, Elementar Gmbh, Frankfurt, Germany). The acid detergent-insoluble crude protein (ADICP) and neutral detergent-insoluble crude protein

(NDICP) levels in each sample were also determined as described by Licitra et al. (1996). Non-fiber carbohydrates (NFC) were calculated as 100 – ash – EE – CP – (aNDF – NDICP) based on the guidelines of NRC (2001). Mineral contents were determined using appropriate AOAC methods (Horwitz, 2000).

In vitro rumen fermentation

Rumen fluid was used for *in vitro* experiments in Hanwoo, which was collected one hour before the morning feed using the stomach tubing method. Rumen pH was measured immediately, using a portable pH meter (Mettler-Toledo AG, Schwerzenbach, Switzerland). After collection, the fluid was filtered through four layers of cheesecloth. The rumen fluid was mixed with McDougall's buffer solution (McDougall, 1948) in a 1 : 4 ratio under anaerobic conditions (Lima et al., 2010) and flushed with O_2-free CO_2 for 20 min. Next, 50 mL of rumen inoculum was dispensed into each 125-mL serum bottle containing 0.5 g of the sample substrate. To maintain anaerobic conditions in the serum bottles, O_2-free CO_2 was injected every 10 sec. The bottles were then sealed with a butyl rubber stopper and aluminum seal and incubated at 39℃ for 24 and 48 h in a shaking incubator at 120 rpm.

After each incubation time point (24 h and 48 h), the serum bottles were removed from the incubator and immediately stored in an ice bucket to prevent further fermentation due to residual heat. After the upper part of the aluminum seal was removed, total gas production was measured by pricking the butyl rubber stopper using a glass syringe (TRUTH 100 mL; Top Syringe Mfg. Co. Pvt. Ltd., Mumbai, India). The rumen inoculum was transferred into a 50-mL conical tube and centrifuged at 6,000 × g for 15 min at 20℃. After centrifugation, the pH was measured using a pH meter (Mettler-Toledo AG, Schwerzenbach, Switzerland). The supernatant was used for the analysis of volatile fatty acids (VFAs) and the concentration of ammonia nitrogen (NH_3-N). The remaining digestion residue was used to calculate *in vitro* dry matter digestibility (DMD).

The feed residues left in the 50-mL conical tubes and 125-mL serum bottle were washed with distilled water, and collected on a 125-mm diameter Whatman filter paper. The liquid part was removed using a vacuum pump, and solid part was used for the dry matter digestion calculation after drying. Filter papers containing the residues were dried at 105℃ for 48 h, and the amount of residue was measured.

The rumen inoculum samples were stored in a deep freezer at - 80℃ until VFA and NH_3-N analyses. After being thawed, 10 mL of sample was mixed with 1 mL of 2% $HgCl_2$ (wt·vol^{-1}) solution and briefly centrifuged at 2,000 × g for 10 min at 4℃ to remove the feed particles. NH_3-N concentration was determined using a Multiplate spectrophotometer (Bio-Rad, Benchmark Plus^{TM}, Tokyo, Japan) at 630 nm (Chaney and Marbach, 1962). VFA concentration was determined using gas chromatography (Varian CP-3800; Varian, Walnut, Creek, CA, USA) as described by Erwin et al (Erwin et al., 1961),

In vivo apparent digestibility

Four Hanwoo steers (32 months old, 562 ± 91 kg initial body weight [BW]) were used for *in vivo* studies. Each animal was housed in a metabolic cage (127 cm width × 250 cm depth × 200 cm height) individually to collect remaining feed and excretions. The animals were fed twice a day (09:00 and 16:00) and given free access to water. According to the Korean Feeding Standard for Hanwoo, experimental diets based on 1.5% BW (DM basis) were supplied by separate feeding, and concentrate mix:forage ratio in the feed was 80:20. All excretions were collected for the measurement of total digestible nutrients. 4 × 4 Balanced Latin Square design was used for this study, with 14 d in one period, 9 d for feed adaptation, and

5 d for fecal collection. The quantity of feed intake was checked. Chemical analysis of the feces was conducted and the metabolic energy was calculated from this value.

Statistical analysis

All data were analyzed using the MIXED procedure in SAS 9.3 software (Statistical Analysis Systems Institute Inc., Cary, NC, USA) using one-way ANOVA and Duncan's multiple range test to compare the means. Statistical significance was considered at $p < 0.05$.

Results and Discussion

Chemical composition

The chemical composition of the four forage types and concentrates are presented in Table 1. Crude protein of the Italian ryegrass, rice straw, oat hay, and timothy hay were 673, 592, 648, 634 $g \cdot kg^{-1}$ DM, respectively. Rice straw had the highest NDF and ADF content among the four forage types, while NFC was highest in Oat hay than other forages. The NDF content of IRG was lower than that of rice straw but greater than that of both oat hay and timothy hay. The NFC content of Italian ryegrass was lower than that of both oat and timothy hay but greater than that of rice straw.

Table 1. Chemical composition of four forage sources and concentrate ($g \cdot kg^{-1}$ dry matter or as stated).

Items	Rice straw	Oat hay	IRG	Timothy hay	Concentratey
Dry matter	936	940	834	903	827
aNDF[z]	673	592	648	634	241
Aid detergent fiber	393	327	339	348	99
Acid detergent lignin	74	56	98	40	18
Nonfiber carbohydrates	159	298	169	221	458
Crude protein (CP)	52	68	183	102	199
Neutral detergent insoluble CP	42	22	99	38	20
Acid detergent insoluble CP	21	13	22	14	8
Ether extract	20	19	13	13	28
Crude ash	138	46	86	68	75
Magnesium	1.7	1.1	1.4	1.2	3.7
Sodium	0.4	2.2	1.6	0.8	4.2
Calcium	3.3	1.8	3.5	1.6	15.7
Phosphorus	1.1	1.4	1.8	2.3	6.1
Gross energy ($Kcal \cdot g^{-1}$)	3.92	4.14	4.17	4.30	4.32

IRG, Italian ryegrass.

[y] Ground corn, 40.2%; Corn gluten feed, 21.2%; Wheat bran, 13.1%; Coconut meal, 4.1%; Rapeseed meal, 4.2%; Palm-kernel meal, 4.2%; Soybean meal, 9.8%; Limestone, 1.9%; Salt, 0.5%; Sodium bicarbonate, 0.5%; Vitamin-mineral mix, 0.3% (Vit. A, 2,650,000 IU; Vit. D3, 530,000 IU; Vit E, 1,050 IU; Niacin, 10,000 mg; Mn, 4,400 mg; Fe, 13,200 mg; I, 440 mg; Co, 440 mg).

[z] aNDFom, neutral detergent fiber analyzed using a eat stable amylase and expressed inclusive of residual ash.

In vitro ruminal fermentation characteristics

The ruminal fermentation characteristics of each forage type are shown in Table 2. The pH of the rice straw after incubation for 24 h (6.95) and 48 h (6.87) was the highest among the four forage types ($p < 0.01$). At 48 h incubation time, the pH of the oat hay (6.72) was the lowest ($p < 0.01$), while IRG (6.79) and timothy hay (6.76) showed moderate pH levels ($p < 0.01$). The total gas production of timothy hay (151.3 $mL \cdot g^{-1}$) and oat hay (168.0 $mL \cdot g^{-1}$) was greater than that of the IRG (140.0 $mL \cdot g^{-1}$) and rice straw (110.0 $mL \cdot g^{-1}$) at 48 h ($p < 0.01$). The pH values showed a negative correlation with total gas production (Table 2). The NH_3-N concentration of IRG (19.69 $mg \cdot dL^{-1}$) was notably higher than that of the other forage types at 48 h ($p < 0.01$). The protein content of IRG (18.3% DM), timothy hay (10.2% DM), oat hay (6.8% DM), and rice straw (5.2% DM) was proportional to the amount of NH_3-N (19.69, 12.72, 10.83, and 9.41 $mg \cdot dL^{-1}$, respectively; Tables 1 and Table 2). The VFA content of oat hay (76.8 $mmol \cdot L^{-1}$) was the highest among the four forage types while that of rice straw (54.1 $mmol \cdot L^{-1}$) was the lowest ($p < 0.01$) at 48 h. IRG (72.6 $mmol \cdot L^{-1}$) and timothy hay (72.7 $mmol \cdot L^{-1}$) showed similar levels of total VFA ($p < 0.01$) at 48 h. The acetic acid concentration of rice straw (66.3%) and oat hay (66.4%) were greater than that of IRG (65.2%) and timothy hay (65.7%; $p < 0.01$) at 48 h. In contrast, IRG and timothy hay had a greater amount of propionic acid than rice straw and oat hay ($p < 0.01$) at 48 h. Therefore, the ratio of acetate acid to propionate acid (A : P) of rice straw and oat hay was higher than that of IRG and timothy hay ($p < 0.01$) at 48 h. IRG showed the lowest A : P ratio among the forage types at 48 h. The *in vitro* DM digestibility (IVDMD) of rice straw was lower than that of the other forage types ($p < 0.01$).

In this study, the NDF content of rice straw was the highest, followed by that of IRG, timothy hay, and oat hay. Rumen cellulolytic bacteria cannot survive at low ruminal pH, and ruminal fiber degradation is significantly decreased under this condition (Chaney and Marbach, 1962; Weimer, 1996).

NH_3-N in rumen inoculum is produced by microbes during the process of amino-acid decomposition (Russell et al., 1992). Ruminants absorb proteins directly as amino acids or use NH_3-N to synthesize microbial proteins, which are then digested and absorbed (Stallings et al., 2009). Both are used as a source of amino acids for the growth and development of rumen microorganisms. In a previous study, high-protein feed was shown to increase the NH_3-N concentration in the rumen (Chaudhary and Parvez, 2012), which is in accordance with the results of our study. IRG had notably higher amounts of crude protein (18.3%) and NH_3-N concentration (19.69 $mg \cdot dL^{-1}$) in our *in vitro* experiments. The crude protein level observed in this study was similar to that of previous studies (17.5%; Burns et al., 2015).

Table 2. Effects of different forage sources on *in vitro* rumen fermentation.

Parameters	Incubation times (h)											
	24						48					
	Rice straw	Oat hay	IRG	Timothy hay	SEM	p-value	Rice straw	Oat hay	IRG	Timothy hay	SEM	p-value
Total gas ($mL \cdot g^{-1}$)	63.30c	104.70b	96.70b	114.70a	2.11	< 0.0001	110.00d	168.00a	140.00c	151.30b	1.56	< 0.0001
pH	6.95a	6.83b	6.86b	6.86b	0.013	0.01	6.87a	6.72c	6.79b	6.76b	0.01	< 0.0001
Ammonia-N ($mg \cdot dL^{-1}$)	6.02c	5.70c	13.90a	8.65b	0.35	< 0.0001	9.41d	10.83c	19.69a	12.72a	0.43	< 0.0001
Total VFA ($mmol \cdot L^{-1}$)	39.10b	56.50a	55.10a	54.10a	0.79	< 0.0001	54.10c	76.80a	72.60b	72.70b	0.48	< 0.0001
Acetate (%)	63.30b	64.80a	64.70a	63.7ab	0.40	< 0.0001	66.30a	66.40a	65.20b	65.70b	0.11	< 0.0001
Propionate (%)	19.70c	20.7b	21.0ab	21.40a	0.20	< 0.0001	19.40b	19.80b	20.90a	20.60a	0.18	< 0.0001
A : P ratio	3.21a	3.13a	3.08ab	2.97b	0.03	< 0.0001	3.42a	3.36a	3.13b	3.19b	0.03	< 0.0001
Butyrate (%)	11.87a	10.61b	8.82c	10.24b	0.19	< 0.0001	9.44b	9.72a	8.11d	9.11c	0.03	< 0.0001
IVDMD (%)	25.50b	41.10a	42.70a	42.10a	1.07	< 0.0001	37.90b	53.40a	50.70a	51.5a	1.05	< 0.0001

SEM, standard error of the means (n = 3); IRG, Italian ryegrass; VFA, volatile fatty acid; A : P ratio, ratio of acetate and propionate; IVDMD, in vitro dry matter digestibility.

a - d: Values with in the same row with different letters are significantly different ($p < 0.05$).

VFA levels indicate the rate of microbial fermentation in ruminants. In the rumen, VFAs are produced by the microbial fermentation of carbohydrates and proteins in the feed (Dijkstra et al., 1993) and are the main energy source (70%) in ruminants. In this study, the total VFAs in oat hay were the highest, followed by timothy hay, IRG, and rice straw. The IVDMD of oat hay was the highest, followed by that of timothy hay, IRG, and rice straw.

In vivo digestibility trials

Results of the *in vivo* digestibility trials are shown in Table 3. The DM, CP, EE, NFC, NDF, and ADF digestibility of the substrate with 80% concentrate with 20% IRG (C80-IRG20) were 72.0, 76.2, 79.9, 86.4, 61.4, and 55.6%, respectively. Of all diets, the energy values of C80-IRG20 yielded the highest values for all treatments. C80-IRG20 had the highest TDN values, followed by the substrates with 80% concentrate with 20% oat hay (C80-OH20), 80% concentrate with 20% timothy (C80-T20), and 80% concentrate with 20% rice straw (C80-RS20). In addition, the digestible energy of C80-IRG20 was the highest in all treatments. The digestible energy to gross energy (DE : GE) ratio was highest for C80-IRG20 and lowest for C80-RS20. The ADF content of rice straw was the lowest, followed by that of timothy hay, IRG, and oat hay. In general, the digestibility of forage showed a negative correlation with ADF (Undersander and Moore, 2012). However, there were no significant differences among treatments. These results can be explained on the basis that the feeding rate of the forage was low and the forage feeding ratio was only 20%; therefore, the effect of forage digestibility on the overall digestibility was low.

Table 3. Effect of different forage sources on nutrient intake, digestibility, and energy.

Items	C80-RS20	C80-OH20	C80-IRG20	C80-T20	SEM	p-value
Nutrient intake ($kg·d^{-1}$)						
DMI	7.08	7.14	7.96	7.67	0.605	0.700
OMI	6.40	6.59	7.27	7.04	0.552	0.680
CPI	1.33	1.35	1.75	1.52	0.122	0.127
EEI	0.21	0.21	0.22	0.21	0.016	0.869
NFCI	3.00	3.25	3.38	3.43	0.267	0.678
NDFI	2.63	2.49	2.92	2.71	0.216	0.594
ADFI	1.35	1.17	1.34	1.34	0.105	0.581
GE ($kcal·d^{-1}$)	33.3	33.9	38.6	36.7	2.891	0.553
Digestibility (%)						
DMD	66.2	70.6	72.0	69.3	1.81	0.21
OMD	67.7	69.7	71.8	68.2	1.89	0.46
CPD	68.1	72.7	76.2	72.4	2.03	0.12
EED	74.0	76.2	79.9	78.1	2.75	0.51
NFCD	88.0	85.1	86.4	85.0	1.90	0.67
NDFD	53.3	56.2	61.4	53.4	3.19	0.31
ADFD	45.3	52.0	55.6	51.5	3.79	0.35
Energy value						
TDN (%)	67.4	70.1	71.9	68.7	1.551	0.272
DE ($Mcal·d^{-1}$)	22.9	24.1	28.4	25.6	2.025	0.322
DE ($Mcal·kg^{-1}$)	2.91	3.06	3.18	3.02	0.074	0.169
DE : GE	0.69	0.71	0.74	0.70	0.018	0.282

SEM, standard error of the means (n = 4); C80-RS20 = concentrate (80%) with rice straw (20%), C80-OH20 = concentrate (80%) with oat hay (20%), C80-IRG20 = concentrate (80%) with Italian ryegrass (20%), C80-T20 = concentrate (80%) with timothy (20%); DMI, dry matter intake; OMI, organic matter intake; CPI, crude protein intake; EEI, ether extract intake; NDFI, neutral detergent fiber intake; ADFI, acid detergent fiber intake; GE, gross energy; DMD, dry matter digestibility; OMD, organic matter digestibility; CPD, crude protein digestibility; EED, ether extract digestibility; NFCD, non-fiber carbohydrate digestibility; NDFD, neutral detergent fiber digestibility; ADFD, acid detergent fiber digestibility; TDN, total digestible nutrients; DE, digestible energy.

a - d: Values with in the same row with different letters are significantly different ($p < 0.05$).

Crude protein digestibility decreased as the ADICP/CP ratios increased, as Weiss (1992) also found. Consequently, the ADICP/CP ratios were as follows: rice straw: 40.4%, oat hay: 19.1%, timothy hay: 13.7%, and IRG: 12.0%. According to Wiess' theory, the CP digestibility of rice straw could be predicted to be the lowest, followed by oat hay, timothy hay, and IRG. However, in the actual experimental results, the CP digestibility of rice straw and IRG was consistent with the theoretical calculation value while the results of oat hay and timothy hay varied. The results showed that the digestibility of rice straw was the lowest, that of oat hay and timothy hay were similar, and the digestibility of IRG was the highest.

Weiss (1992) suggested that true digestible NFC digestibility (NFCD) was 98%. We found 85 - 88% NFCD in all treatments, indicating that NFCD had the highest digestibility of all nutrients. These differences between the findings of Weiss and ours may be due to the results of the apparent digestibility *in vivo* and the true digestibility (as derived from an equation). Tebbe et al. (2017) reported that in 36 experiments comprising 214 cattle, the average digestibility of NFC was 78.4 - 89.8% in the 10^{th} - 90^{th} percentile (Tebbe et al., 2017). This is similar to the results of the present study.

A previous study showed that forages with high lignin content showed low NDF digestibility. However, this study showed that lignin content has no effect on NDF digestibility. In other studies, the NDF digestibility was not only found to be affected by lignin content but also by ester and ether linkages, which vary with maturity, varietal genetics, and agronomic factors (Raffrenato et al., 2017).

Although the GE values of IRG were lower than those for timothy hay and oat hay, the DE values and DE:GE ratio of IRG were the highest among the forage types. These results indicate that the metabolic utilization rate of IRG was the highest among the treatments. Thus, we conclude that IRG is the best forage source among the four types analyzed in this study.

Conclusions

Italian ryegrass as a level of energy efficiency similar to that of oat hay and timothy hay, even though IRG is more fibrous. In the results of the overall experiment, high NH_3-N concentration in *in vitro* fermentation and high CP digestibility in *in vivo* experiments indicate that IRG meets protein requirements. IRG could be a good forage source owing to its high protein content and energy efficiency. Rice-straw silage is widely used as a forage source in Korea; however, it is generally thought to be low in nutrient content (Conrad, 1996). The results of the present study also indicate that rice straw had a lower nutritional value than the other forage sources analyzed. In addition, oat hay and timothy hay can be used as replacements for rice straw. In conclusion, IRG contains high-protein feed resources and could be a potential substitute for imported forage.

Conflicts of Interst

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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