Change in Nitrogen Fractions and Ruminal Nitrogen Degradability of Orchardgrass Ensiled at Various Moisture Contents and the Subsequent Effects on Nitrogen Utilization by Sheep

H. V. Nguyen[1], M. Kawai, J. Takahashi and S. Matsuoka*
Department of Animal Science, Obihiro University of Agriculture and Veterinary Medicine
Obihiro, Hokkaido 080-8555, Japan

ABSTRACT : The effect of various moisture contents of fresh forage on the change in nitrogen (N) fractions, *in vitro* ruminal N degradability, and the subsequent N utilization of silage in sheep were evaluated. Orchardgrass (*Dactylis glomerata L.*) with high (HM, 76%), medium (MM, 65%) and low (LM, 40%) moisture contents were ensiled into silos of 120 L capacity for 120 days. A nitrogen balance trial was conducted using a 4×4 Latin square design consisting of four dietary treatments (i.e. fresh forage, HM, MM and LM silages) and four wethers. With respect to N fractions, fraction 1 (buffer solution soluble N), fraction 2 (buffer solution insoluble N-neutral detergent insoluble N), fraction 3 (neutral detergent insoluble N-acid detergent insoluble N), and fraction 4 (acid detergent insoluble N) were determined. The proportion of fraction 1 in silages tended to decrease, while the *in vitro* ruminal degradability of insoluble N increased ($p<0.05$) with lower moisture contents at ensiling. Consequently, nitrogen utilization in sheep tended to improve as the moisture content of ensiled grass was decreased, with a negative correlation ($p<0.01$) between urinary N and the *in vitro* ruminal degradability of insoluble N. The averaged N retentions for HM, MM, and LM silage treatments were 59, 73 and 79% of that for fresh forage, respectively. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 9 : 1267-1272)***

Key Words : Orchardgrass, Moisture Contents, Ensiling, Nitrogen Fractions, Ruminal Degradability, Nitrogen Utilization

INTRODUCTION

It is well known that when fresh forage is ensiled, fermentation results in quality changes that alter the feed value of silage as compared to fresh forage. There are two major changes which can occur during ensiling process: sugars are broken down into organic acids, while proteins are broken down into ammonia and other non-protein compounds.

The extent of true protein breakdown varies owing to several factors such as plant species, rate and extent of pH changes, moisture content and temperature, but it may reduce the protein content by 50 to 60% of original herbage, even in well-preserved silages (McDonald et al., 1991). Such changes in the nitrogenous components adversely affect the subsequent utilization of N in ruminants (McDonald et al., 1991; Nguyen et al., 2004a, b).

Generally, the optimum moisture content for precision-chopped silage is about 65% (Ensminger et al., 1990; Horrocks and Vallentine, 1999), and the degree of hydration will facilitate the fermentation process and usually helps to eliminate oxygen from the silage mass during packing. The impact of moisture content on proteolysis in silage has been studied fairly extensively (Merchen and Satter, 1983; McDonald et al., 1991; Chamberlain and Wilkinson, 1996). However, very few data are available that show the effect of various moisture contents at ensiling on the change in N fractions of forage protein.

Objectives of this study were therefore to clarify the extent of change in N fractions and in vitro ruminal N degradability of orchardgrass silage as ensiled at high (76%), medium (65%), and low (40%) moisture contents respectively; and to evaluate the subsequent influence on N balance in sheep.

MATERIALS AND METHODS

Silage preparation

A first cut orchardgrass (*Dactylis glomerata L.*) was harvested in the early heading stage from the experimental farm of Obihiro University of Agriculture and Veterinary Medicine (Hokkaido, Japan) during mid-June. After mowing, the grass was left wilting on the field for about 3 h and then picked up using a precision chop forage harvester (theoretical chop length 2 cm). The entire harvested material (about 1 tonne) was thoroughly mixed to avoid any field effects that may have been present during harvest, and then divided into four equal portions. The first portion was stored immediately at -15°C for later use as fresh forage. The second portion was immediately ensiled in three plastic silos of 120 L capacity. The third and fourth portion were allowed to wilt under natural sunlight until the moisture content of the grass reached about 65 and 40%, respectively.

* Corresponding Author: S. Matsuoka. Tel: +81-155-49-5422, Fax: +81-155-49-5462, E-mail: matsuoka@obihiro.ac.jp
[1] Nguyen Huu Van, Faculty of Animal Science, Hue University of Agriculture and Forestry, 24 Phung Hung st, Hue city, Vietnam.
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Table 1. Chemical composition of fresh forage and silages*

	Fresh forage	Silages			
		HM	MM	LM	SE
Moisture content (%)	75.7	76.4[a]	64.5[b]	39.6[c]	0.11
Crude protein (% DM)	13.8	13.3	13.4	13.6	0.19
Neutral detergent fibre (% DM)	53.1	50.4[c]	51.6[b]	52.5[a]	0.03
Acid detegent fibre (% DM)	30.8	30.2[c]	31.2[b]	31.9[a]	0.11
Acid detergent lignin (% DM)	10.0	10.2[b]	10.7[a]	11.0[a]	0.13
Water soluble carbohydrates (% DM)	6.4	1.5[b]	1.6[b]	4.9[a]	0.06
Gross energy (MJ/kg DM)	17.7	16.5[c]	16.8[b]	17.3[a]	0.02

* Values are mean of three silos except for fresh forage.
[a,b,c] Means in the same row with unlike superscripts are significantly different ($p<0.05$).

before being packed into the three silos. All silos were opened after 120 days of ensiling. Representative sample from each silo was taken, and the remaining silage of the three silos from each treatment was mixed and stored in freezers at -15°C until being fed to sheep in N balance trial. As described above, all the fresh forage and silages were stored in the same freezer, this helps offset any differential effects that freezing may have caused between silage and fresh forage. Chemical composition of fresh forage and subsequent silages are shown in Table 1.

Fractionation of forage nitrogen

The fractionation scheme used was basically from the Cornell Net Carbohydrate and Protein System (Sniffen et al., 1992) where feed proteins are partitioned into five fractions (A, B_1, B_2, B_3 and C). However, in the present study A and B_1 were not separated since the proportion of B_1 is a very small part of the total nitrogen of forage and it is also assumed to be totally degraded in the rumen as is the A fraction (Krishnamoorthy et al., 1982; Sniffen et al., 1992). Therefore, the fractionation of forage nitrogen was as follows: Fraction 1, Fraction 2, and Fraction 3, calculated by subtracting buffer solution insoluble nitrogen (BSIN) from total nitrogen (TN), neutral detergent insoluble nitrogen (NDIN) from BSIN, and acid detergent insoluble nitrogen (ADIN) from NDIN, respectively; and Fraction 4 is ADIN.

Estimation of ruminal degradability of forage nitrogen

In vitro procedure of Coblentz et al. (1999) was applied to estimate the degradability of forage nitrogen in rumen. Actinase E (Kaken Pharmaceutical Inc., Tokyo, Japan), a protease enzyme made from *Streptomyces griseus*, that contains 10^6 activity units/g of solid was used in this study. Enzyme solution was prepared freshly in a borate-phosphate buffer (pH 8.0). Samples containing 15 mg of nitrogen were put into 80 ml capacity glass bottles with screw caps and incubated for 1h at 39°C in a 40 ml borate-phosphate buffer. Following the 1h buffer incubation, 10 ml of prepared protease solution, containing 33 activity units/ml was added to each bottle yielding final enzyme activity concentration of 6.6 units/ml in the incubation medium. The bottles were then continuously incubated for 4 h at 39°C. After incubation, samples were filtered immediately through Toyo No.5A filter paper (Toyo Roshi Kaisha, Ltd., Japan), and the N content of each residue was determined by Kjeldahl's method.

Nitrogen balance trial

The prepared fresh forage and three silages (i.e. low moisture (LM), medium moisture (MM), and high moisture (HM)) were fed to four wethers in a 4×4 Latin square design. The animals were kept in metabolism cages with free access to water and a mineral block (Nihonzenyaku Inc., Tokyo, Japan). The feeds were offered to the animals at maintenance (2% of body weight in dry matter basis), twice daily at 08:00 and 17:00. Each period of the experiment was 12 days, including 7 days of adjustment to the respective dietary treatments and 5 days of sample collection. Fecal and urinary samples were immediately preserved in freezers at -15°C until analysed.

Chemical analyses

Fresh forage and silage were dried for 24 hours in a freeze-drier (VD-40/TAITEC, Japan) to determine dry matter (DM), being left in the experimental room for 7 days to prepare air-dried samples. Feces were dried in a forced air oven at 60°C for 72 h to prepare air-dried samples. DM of feces were then determined by using an oven at 135°C for 2 h. The prepared air-dried samples were ground by a Wiley mill (1 mm screen) for subsequent analyses. Neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) were determined as described by Van Soest et al. (1991). Neutral and acid detergent residues were analyzed for N to determine the NDIN and ADIN, respectively. BSIN was determined by the method of Krishnamoorthy et al. (1982). Nitrogen (N) was determined by Kjeldahl's method using an automatic apparatus (UDK 130 D/VELP Scientifica, Italia) and crude protein (CP) calculated as N×6.25. Water soluble carbohydrate (WSC) was determined colorimetrically using the Anthrone method of Masaki (1971). The silage extract was prepared for

Table 2. Fermentation characteristics of silages*

	Silages			
	HM	MM	LM	SE
pH	3.96[c]	4.05[b]	4.49[a]	0.01
Lactic acid (% DM)	7.1[a]	5.5[b]	2.5[c]	0.12
Acetic acid (% DM)	0.1[b]	0.1[b]	0.2[a]	0.02
Propionic acid (% DM)	-	-	-	-
Butyric acid (% DM)	-	-	-	-
Ammonia-N (% TN)	6.4[a]	4.1[b]	3.0[c]	0.22
Water soluble carbohydrates (% DM)	1.5[b]	1.6[b]	4.9[a]	0.06

* Values are means of three silos.
[a, b, c] Means in the same row with unlike superscripts are significantly different ($p<0.05$).

Table 3. Nitrogen fractions (% of TN) of fresh forage and silages*

	Fresh forage	Silages			
		HM	MM	LM	SE
Fraction 1	26.4	48.0[a]	44.7[a]	32.9[b]	0.99
Fraction 2	41.1	31.4	28.7	28.0	0.88
Fraction 3	21.3	10.8[c]	16.7[b]	27.8[a]	1.03
Fraction 4	11.2	9.8[b]	10.0[b]	11.3[a]	0.28

Fraction 1 = 100-BISN; Fraction 2 = BSIN-NDIN; Fraction 3 = NDIN-ADIN; Fraction 4 = ADIN.
* Values are means of three silos except for fresh forage.
[a, b, c] Means in the same row with unlike superscripts are significantly different ($p<0.05$).

measuring pH (pH meter/Horiba F-7AD), lactic acid (Barker and Summerson, 1961), volatile fatty acids (VFA; capillary column gas chromatography/Shimadzu CG-14A), and ammonia nitrogen (Conway and O'Malley, 1942). Gross energy (GE) content in feed was determined by using an adiabatic bomb calorimeter (CA-4P, Shimadzu, Japan).

Statistical analysis

The difference among treatments was analyzed by analysis of variance (ANOVA) according to the General Linear Model (GLM) procedures of Statistical Analysis System (SAS) Institute Inc. (1997) with mean separation by Duncan's multi-range test. Statistical significance of differences was accepted at $p<0.05$. Correlation coefficients between N balance parameters (number of data set was sixteen) with N fractions and *in vitro* ruminal N degradability were also computed using procedures of SAS Institute Inc. (1997).

RESULTS

The fermentation characteristics of silages are shown in Table 2. The pH increased ($p<0.05$) while the lactic acid and NH_3-N content decreased ($p<0.05$) with reducing moisture content of ensiled grass. Only a very small amount of acetic acid was found, and there was no detectable concentration of propionic acid or butyric acid in any silages. After 120 days of ensiling, the WSC content remaining in LM silage was significantly higher ($p<0.05$) than those in MM and HM silages.

Table 4. *In vitro* ruminal degradability (%) of total N and insoluble N of fresh forage and silages*

	Fresh forage	Silages			
		HM	MM	LM	SE
Total N degradability	66.4	69.9[ab]	71.4[a]	67.4[b]	0.72
Insoluble N degradability	54.3	42.1[c]	48.2[b]	51.4[a]	0.45

Total N degradability = (TN-undegraded N)/TN×100.
Insoluble N degradability = (BSIN-undegraded N)/BSIN×100.
* Values are means of three silos except for fresh forage.
[a, b, c] Means in the same row with unlike superscripts are significantly different ($p<0.05$).

Table 5. Nitrogen balance in sheep fed on fresh forage and silages*

	Fresh forage	Silages			
		HM	MM	LM	SE
N intake (g/day)	20.7[a]	19.7[b]	19.7[b]	20.0[b]	0.13
Fecal N (% of N intake)	30.1[b]	35.0[a]	33.0[ab]	34.4[ab]	0.99
Urinary N (% of N intake)	40.4[b]	47.7[a]	45.5[ab]	42.4[ab]	1.46
Retained N (% of N intake)	29.5[a]	17.3[b]	21.5[ab]	23.2[ab]	1.87

* Values are mean of four sheep in the 4 4 Latin square design.
[a, b, c] Means in the same row with unlike superscripts are significantly different ($p<0.05$).

Nitrogen fractions of the fresh forage and silages are shown in Table 3. The proportion of fraction 1 in silages was higher than that in the fresh forage, and significantly increased ($p<0.05$) with higher moisture contents of ensiled grass. In contrast, the proportion of fraction 2 in silages was lower than that in the fresh forage, and there was no significant difference among silages. The proportion of fraction 3, with the exception of LM, was also lower in silages than in the fresh forage. However, among silages this value remarkably increased ($p<0.05$) as the moisture content of ensiled grass was decreased. The proportion of fraction 4 in either of silage treatments was not much changed after ensiling. A small but significant change in fraction 4 was observed among silage treatments.

In vitro degradability of total N and of insoluble N of fresh forage and silages are shown in Table 4. All silages showed a higher total N degradability but a lower insoluble N degradability compared with the fresh forage. Among the silages, total N degradability of LM was lowest, and was significantly lower ($p<0.05$) than that of MM silage. Insoluble N degradability increased remarkably ($p<0.05$) with reducing moisture content.

Nitrogen balance parameters in sheep fed on fresh forage and silages are shown in Table 5. N intake was slightly lower ($p<0.05$) for silages than for the fresh forage, however the differences among four dietary treatments

Table 6. The correlation coefficients between N balance parameters with N fractions and *in vitro* ruminal N degradability

	Nitrogen fractions				Degradability	
	1	2	3	4	Total N	Insoluble N
Fecal N	0.406	-0.488	-0.130	-0.272	0.287	-0.431
Urinary N	0.629**	-0.363	-0.514*	-0.597*	0.539*	-0.624**
Retained N	-0.690**	0.540*	0.452	0.592*	-0.558*	0.700**

* $p<0.05$; ** $p<0.01$.

Nitrogen balance parameters are calculated as percent of nitrogen intake.

appeared to be negligible. The percentage of fecal N was higher for silages than for the fresh forage, with a significant difference ($p<0.05$) between HM silage and the fresh forage. There was no significant difference among the silage treatments. With respect to the percentage of urinary N, a similar trend was observed between silages and the fresh forage. There was a declining tendency of urinary N for silages with reducing moisture content, though non-significant ($p>0.05$). The percentage of N retention in sheep was lower for silages than for the fresh forage, with a significant difference ($p<0.05$) between HM silage and the fresh forage. It tended to increase with reducing moisture content of ensiled grass, though non-significant ($p>0.05$).

The relationship between N balance parameters with N fractions and *in vitro* ruminal N degradability is shown in Table 6. There was no significant correlation ($p>0.05$) between fecal N with N fractions and N degradability. Urinary N was positively correlated with fraction 1 ($p<0.01$) and total N degradability ($p<0.05$), while negatively correlated with fraction 3 and 4 ($p<0.05$) and insoluble N degradability ($p<0.01$). Retained N was negatively correlated with fraction 1 ($p<0.01$) and total N degradability ($p<0.05$), while positively correlated with fraction 2 and 4 ($p<0.05$) and insoluble N degradability ($p<0.01$).

DISCUSSION

The three silages were well preserved as indicated by relatively low contents of NH_3-N and the absence of butyric acid (Woolford, 1984; McDonald et al., 1991; Chamberlain and Wilkinson, 1996). These results are in agreement with Chamberlain and Wilkinson (1996) who have shown that the main effects of wilting on the composition of the silage are a restriction of lactic fermentation and an increase of residual water soluble carbohydrates in the silage. Other studies (McDonald, 1976; Gordon et al., 2000; Yahaya et al., 2002), where orchardgrass and ryegrass were ensiled at various moisture contents, have also shown similar observations.

Fraction 1 consists of mostly NPN (NH3, amines, amides, amino acids, peptides) and a small amount (approximately 5% of total soluble nitrogen) of true protein such as globulins, and some albumins (Krishnamoorthy et al., 1982; Sniffen et al., 1992). It was reported that about 10-25% (Hergaty and Peterson, 1973; Oshima and McDonald, 1978) of the N in fresh herbages is present in non-protein compounds. In the present study, fraction 1 in fresh forage was 26.4%, being slightly higher than those values because it may include additional NPN produced during wilting (Marsh, 1979; McDonald et al., 1991). Moreover, the higher proportion of fraction 1 in silages than in the fresh forage was thought to be due to the breakdown of true protein in fraction 2 and 3 during the ensiling process (McDonald et al., 1991; Nguyen et al., 2004a, b). Among silages, the change in the proportion of fraction 1 was in agreement with Chamberlain and Wilkinson (1996) who have suggested that the proportion of soluble nitrogen is likely to be higher in extensively fermented silages than in those which have undergone a restricted fermentation. Similarly, Merchen and Satter (1983) reported that soluble nitrogen in the total nitrogen of alfalfa silages increased with higher moisture content of ensiled material.

Fraction 2 consists of most albumins and glutelins. Fraction 3 is prolamines, of which part is cell wall protein (Sniffen et al., 1992). After mowing, rapid proteolysis takes place resulting in a part of these proteins being hydrolysed. When herbage is ensiled, either directly or after wilting, proteolysis continues (McDonald et al., 1991). In agreement, the present study showed a remarkably decreased proportion of fraction 2 and fraction 3 in HM silage compared with the fresh forage. Nguyen et al. (2004a, b) have also reported similar observations on orchardgrass and alfalfa silages. There was no significant difference in fraction 2 among silage treatments, while there was a significant increase of fraction 3 with lowering moisture content. Although reports on the effect of moisture content during ensiling have been inconsistent (McDonald et al., 1991), our results supported those of Henderson et al. (1982) who found that protein-N content of silages increased progressively with increasing DM content. The high proportion of fraction 3 in LM silage was probably due to proteolysis occurring during the wilting period before the grass was ensiled. Janicki and Stallings (1988) have shown a high proportion of fraction 3 (29.8%) in orchardgrass hay (14% moisture content). It is reasonable to assume that the sunlight and ultraviolet rays at the time of wilting may alter properties of protein, resulting in an increased formation of complex between proteins and plant cell wall carbohydrates. The results in this study showed that the higher proportion of fraction 3 was mainly attributed to the higher neutral

detergent insoluble N content in LM silage than those in MM and HM silages.

Fraction 4 is acid detergent insoluble N that contains proteins associated with lignin, tannin-protein complex and Maillard products (Sniffen et al., 1992). Results of the present study were in agreement with Nguyen et al. (2004a) who have shown that there was no great change in proportion of fraction 4 in orchardgrass and alfalfa after ensiling. Although the proportion of fraction 4 in HM and MM was significantly lower ($p<0.05$) than that in LM silage, the difference appeared to be very small among silage treatments.

In the rumen, the degradation rates are rapid, intermediate and slow for fraction 1, fraction 2 and fraction 3, respectively (Licitra et al., 1996). Fraction 1 is completely degraded, fraction 2 is partially degraded, and fraction 3 is partially degraded but to a lesser extent compared with fraction 2, while fraction 4 is not degraded in the rumen (Krishnamoorthy et al., 1982; Sniffen et al., 1992). The explanation for the lower total N degradability for LM silage in this study is due to lower proportion of fraction 1 in LM silage than those in HM and MM silages. In contrast, the increased insoluble N degradability is probably attributable to the increased proportion of fraction 3 with reducing moisture content of the ensiled grass. Similarly, Nguyen et al. (2004a, b) have reported a higher insoluble N degradability for silages containing higher proportions of fraction 3.

The lower N retention for silage than for the fresh forage could be due mainly to the above mentioned changes in N of grass during ensiling. Because a high proportion of N of naturally fermented silages is in a non-protein, and soluble form, they would be extensively and rapidly converted to ammonia by rumen microbes. If the ammonia is not utilized within a short time or assimilated into microbial protein, it may move across the rumen wall into the blood stream. In the liver it is converted into urea and though some may be recycled to the rumen from the blood stream and via the saliva, much would be lost to the urine. The present study indicated that urinary N was positively correlated with the proportion of fraction 1 and total N degradability, while retained N was negatively correlated with these two parameters.

In general, the soluble nitrogen in silage can supply ammonia-N for rumen microbes for a short time after feeding because it is rapidly degraded in the rumen. Thereafter, insoluble N, which is slowly degraded in the rumen, can be the main source of ammonia-N supply for growth of rumen microbes. The increase of ammonia-N supply during late phase might increase the efficiency of N utilization by rumen microbes, and subsequently by the ruminant. In the present study, although either fraction 2 or fraction 3 did not show a strong relationship with urinary N and retained N, their combined proportion (i.e. fraction 2+fraction 3) showed a significantly negative correlation ($r = -0.630$; $p<0.01$) with urinary N and a signifcantly positive correlation ($r = 0.696$; $p<0.01$) with retained N. Normally, fraction 4 is negatively correlated to retained N because it is not degraded in the rumen and not digested in the lower gut. However, its proportion in total N is small, thereby a reverse relationship was found in the present study.

It is necessary to note that soluble N converted to ammonia can only be used as a N source for rumen microbial synthesis if there is an available carbohydrate to provide the carbon skeletons required for microbial protein synthesis. Nguyen et al. (2003) have demonstrated that the compensation of glucose before feeding with an equivalent amount to the WSC lost during fermentation improved N utilization of orchardgrass silage in sheep. In the present study, the increase of N retention for LM silage was probably not only due to the decrease of fraction 1 and increase of fraction 3 but also a result of a high WSC content which remained after ensiling (Table 1).

In conclusion, the proportion of fraction 1 was decreased while the percentage of insoluble N degradability was increased with reducing moisture content of ensiled grass, resulting in the improvement of N utilization by animals. The averaged N retention for HM, MM, and LM silage treatments were 59, 73 and 79% of that for the fresh forage, respectively.

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