

Effects of Addition of *Lactobacillus plantarum* and *Enterococcus faecium* Inoculants to High-Nitrogen Fertilized Timothy (*Phleum pratense* L.) on Fermentation, Nutritive Value, and Feed Intake of Silage

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Abstract

The objective of this study was to examine the effects of addition of inoculant to high-nitrogen (N) fertilized timothy on fermentation, nutritive value, and feed intake of silage. The silage of timothy cultivated with a N fertilizer rate at two levels (high level (H), standard level (S)) was prepared with (SI, HI) or without (SC, HC) an inoculants. The CP content of H increased by 38 g·kg⁻¹ DM compared with that of S, and the WSC and ADF contents decreased compared with those of S. Regarding the fermentation of silage, the pH and NH₃-N ratio was significantly lower in the silage with inoculant (SI and HI), showing improvement of the fermentation compared with those of the silage without the addition (SC and HC). The CP content was significantly higher in HC and HI than in SC and SI, and the NDF content was the lowest in HI among the four treatments. The CP digestibility of HC and HI was significantly higher than those of SC and SI. The EE digestibility of SI was significantly higher than that of SC, and that of HI was significantly higher than that of HC. The DCP

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contents were significantly higher in HC and HI than in SC and SI. The TDN content was the lowest in SC and highest in HI. The DCP intakes of HC and HI were significantly higher than those of SC and SI. When the N fertilizer rate was increased, the DCP content and DCP intake of the silage increased, and the addition of inoculant improved the fermentation and increased the TDN content.

Keywords

DCP Intake, Fermentation, High-Nitrogen Fertilizer Rate, *Lactobacillus plantarum*, Timothy Silage

1. Introduction

In Japan, roughage given to dairy cattle is mainly grass, and it is generally stored in silage. The milk yield per cow has recently increased markedly, in association with which the amount of concentrates given to them has increased to meet the nutrient intake, elevating the dependence on concentrates. Since concentrates are mostly imported, the feed cost ratio to the operating expenses can be reduced by increasing the self-supplied feed rate.

The crude protein (CP) content of temperate grass silage produced in Japan is low (8.8 - 13.9 g·kg⁻¹ DM) [1] and insufficient to play the role of a protein source, which is a factor increasing the amount of concentrates required. Methods to increase the CP content of grass silage, such as increasing nitrogen (N) fertilizer rate [2] and the ratio of legumes in mixed seeding [3], have been developed into practical techniques and their use has spread. Many research accomplishments have been achieved for N fertilizer rate-based grass cultivation methods. It has been reported that, for grass cultivation in Hokkaido, fertilizer application of N at a level exceeding the standard increased the CP content of the grass but reduced the increase efficiency of the dry matter yield [4]. Moreover, inappropriate and excess N fertilizer rate decreases the water-soluble carbohydrate (WSC) content of grass [5] [6], increases the nitrate N content [7] [8], increases residual N in soil [9]-[11], and induces loss of soil N [12].

On the other hand, it has been reported that the fermentation deteriorates in silage prepared with high-CP orchardgrass cultivated with high N fertilizer rate [13]. Reduction of fermentation is due to increased ammonia production through degradation of CP in the fermentation process [14]. Accordingly, a storage technique that does not reduce the fermentation of high-CP grass silage is required. The promotion of lactic acid fermentation is the best approach to prevent reduction of the fermentation of silage. Inoculant plays the major role in the promotion of lactic acid fermentation, and improvement of the fermentation of grass silage by adding inoculant has been reported by many researchers [15]. However, only a few studies on the fermentation of silage prepared with high-CP grass have been performed. If high-N fertilizer rate increases the CP content of grass materials and the fermentation of silage is improved, the silage-derived CP intake of dairy cattle increases, with which the concentrates-derived CP intake can be decreased.

In this study, we cultivated timothy with a N fertilizer rate at two levels to prepare grass materials with two levels CP contents. Silage of these grass materials was prepared with the addition of an inoculant, and the fermentation was compared. In addition, a silage feeding trial was performed, in which sheep were fed with the silage, and the feeding value was compared with regard to the digestibility, nutritive value, and feed intake of the silage.

2. Materials and Methods

2.1. Grass Materials and Fertilizer Rate

For the grass material, a late variety of timothy, “Hokusyū”, was used. It was seeded on July 26, 2006, in a field of Tenpoku Substation of Kamikawa Agricultural Experiment Station, Hokkaido Research Organization. The seeding density was 20 kg·ha⁻¹. The N fertilizer rate was 160 and 240 kg·ha⁻¹ per year, being designated as standard (S) and high (H) level applications, respectively. The fertilizer rate distribution was two-thirds on May 7, 2007, and one-thirds on June 25, 2007. The field area for each fertilizer rate was 100 m² (10 m × 10 m), and three replicates each of S and H applications were randomly set. The samples of grass material in the three replicates were mixed and subjected to grass material analysis. For the N fertilization, urea was used. The total annual phosphoric acid and potassium fertilizer rate were 60 and 150 kg·ha⁻¹, respectively, in all treatments. For the phosphoric acid and potassium fertilizations, superphosphate of lime and potassium sulfate were used,

respectively. The distribution and timing of fertilization were the same as for the N fertilization. Grass was harvested on June 22, 2007.

2.2. Silage Preparation

Silage was prepared with (SI and HI) and without (SC and HC) an inoculant. In each treatment, the unwilted grass material was cut into 1 - 4 cm lengths and ensiled in 220 L FRP silos. Three replicates were prepared. For the inoculant, 11F25 (*Lactobacillus plantarum* and *Enterococcus faecium*) of Pioneer Hi-Bred Japan Co. Ltd. (Tokyo, Japan) was used. The preparation was added at $1 \text{ mg} \cdot \text{kg}^{-1}$ fresh grass, and the number of viable microorganisms was $1.0 \times 10^5 \text{ cfu} \cdot \text{g}^{-1}$ fresh grass. The silage was subjected to evaluation of the fermentative quality and feed and digestion trials after storage.

2.3. Feed and Digestion Trials

Feed and digestion trials were performed using four male Corriedale cross sheep at the Tokyo University of Agriculture (Abashiri, Hokkaido, Japan). The sheep weighed $77.3 \pm 9.3 \text{ kg}$ (mean \pm standard deviation). The trials were performed between August 2, 2007, and September 18, 2007. Both trials were performed by maintaining sheep in a metabolic cage capable of separating feces and urine, employing the total feces collection method. Four sheep were assigned to each treatment, and the 4×4 Latin-square method was employed for analysis. One trial period was comprised of a 7-day adaptation period and 5 day collection period, lasting 12 days in total. The feed and digestion trials were performed after approval by the Tokyo University of Agriculture Animal Care and Use Committee. The animals were given free access to feed, water, and mineral blocks throughout the trial period.

2.4. Chemical Analysis

Air-dried samples of the grass materials, silage, and feces were prepared by draught drying at 60°C for 48 hours and then analyzed. Silage extract was prepared by homogenizing 50 g of fresh silage with 100 mL of distilled water. The dry matter (DM), CP, ether extract (EE), and gross energy (GE) contents of the grass materials, silage, and feces were measured using the AOAC method [16], and acid detergent fiber (ADF), neutral detergent fiber (NDF) content were measured using the method reported by Van Soest *et al.* [17]. The water-soluble carbohydrate (WSC) and nitrate nitrogen ($\text{NO}_3\text{-N}$) contents of the grass materials were measured using the methods reported by Deriaz [18] and Morimoto *et al.* [19], respectively.

The glucose, fructose, and sucrose contents were measured using high-performance liquid chromatography (LC-10AT; Shimadzu Co. Ltd., Kyoto, Japan). The sample was immersed in 80% ethanol for 24 hours or longer. Extracts was decompression concentrated under diminished pressure and filtered by Sep-PakC18 (Nihon Waters K.K., Tokyo, Japan). For the HPLC column, Shodex NH2P-50 4E (Showa Denko, Kawasaki, Japan) chromatographic column was used. Analysis was performed under the following conditions: mobile phase, $\text{H}_2\text{O}/\text{CH}_3\text{CN} = 25/75$; flow rate, $1.0 \text{ mL} \cdot \text{min}^{-1}$; refractive index detector, RI; and column temperature, 35°C .

The pH of silage was measured using a glass electrode pH meter (HM-25G; Toa DKK Co. Ltd., Tokyo, Japan), the lactic acid content was measured using the Barker-Summerson method [20], the volatile fatty acid (VFA) content was measured using gas chromatography (GC-14A; Shimadzu Co. Ltd., Kyoto, Japan) [21], and the ammonia nitrogen ($\text{NH}_3\text{-N}$) content was measured using the steam distillation method [22]. The V-score was calculated from the acetic acid, butyric acid contents, and the ratio of the $\text{NH}_3\text{-N}$ content to the total nitrogen content ($\text{NH}_3\text{-N}$ ratio) using the score table of a new evaluation method [23].

2.5. Statistical Analysis

All data were subjected to analysis using SAS statistical software (SAS Institute Japan Ltd., Tokyo, Japan). Two-way ANOVA was used to test the effects of the main factors (N fertilizer rate and addition of inoculant) and their interactions on fermentation, chemical composition, digestibility, nutritive value, and feed intake of silage.

3. Results

3.1. Chemical Compositions of Grass Materials

The chemical compositions of the grass materials are shown in **Table 1**. The CP content was higher by $38 \text{ g} \cdot \text{kg}^{-1}$

Table 1. Chemical composition of grass materials.

Item	S	H
DM (g·kg ⁻¹)	124	133
OM(g·kg ⁻¹ DM)	903	913
CP (g·kg ⁻¹ DM)	136	174
EE (g·kg ⁻¹ DM)	35	41
ADF (g·kg ⁻¹ DM)	360	323
NDF (g·kg ⁻¹ DM)	581	581
WSC (g·kg ⁻¹ DM)	83	61
Glucose (g·kg ⁻¹ DM)	27.4	29.7
Fructose (g·kg ⁻¹ DM)	20.1	22.0
Sucrose (g·kg ⁻¹ DM)	1.3	1.6
NO ₃ -N (g·kg ⁻¹ DM)	1.6	2.2
GE (MJ·kg ⁻¹ DM)	20.2	20.3

S: standard level nitrogen fertilizer rate (160 kg·ha⁻¹); H: high level nitrogen fertilizer rate (240 kg·ha⁻¹); DM: dry matter; OM: organic matter; CP: crude protein; EE: ether extract; ADF: acid detergent fiber; NDF: neutral detergent fiber; WSC: water soluble-carbohydrate; NO₃-N: nitrate nitrogen; GE: gross energy.

DM in H (174 g·kg⁻¹ DM) than in S (136 g·kg⁻¹ DM), and the ADF content was lower by 37 g·kg⁻¹ DM in H (323 g·kg⁻¹ DM) than in S (360 g·kg⁻¹ DM). The WSC content was lower by 22 g·kg⁻¹ DM in H (61 g·kg⁻¹ DM) than in S (83 g·kg⁻¹ DM). In WSC, the glucose and fructose contents were high and the sucrose content was low in both grass materials. The NO₃-N content of H was 2.2 g·kg⁻¹ DM, being higher than that of S (1.6 g·kg⁻¹ DM).

3.2. Fermentation and Chemical Compositions of Silage

The fermentation and chemical compositions of the silage are shown in **Table 2**. The pH and NH₃-N ratio were significantly lower in SI (4.46 and 83.9 g·kg⁻¹ TN, respectively) than in SC (4.75 and 124.6 g·kg⁻¹ TN, respectively) ($P < 0.01$), and significantly lower in HI (4.51 and 82.7 g·kg⁻¹ TN, respectively) than in HC (4.75 and 105.5 g·kg⁻¹ TN, respectively) ($P < 0.01$). The lactic acid content and V-score were significantly higher in SI (34.3 g·kg⁻¹ DM and 91.9, respectively) than in SC (15.9 g·kg⁻¹ DM and 77.8, respectively) ($P < 0.01$), and significantly higher in HI (35.3 g·kg⁻¹ DM and 92.0, respectively) than in HC (24.1 g·kg⁻¹ DM and 85.1, respectively) ($P < 0.01$).

No significant difference was noted in the DM content among the four treatments, and it ranged from 134 to 138 g·kg⁻¹. No significant difference was noted in the CP content between SC (138 g·kg⁻¹ DM) and SI (134 g·kg⁻¹ DM) and between HC (171 g·kg⁻¹ DM) and HI (165 g·kg⁻¹ DM). The CP contents of HC and HI were significantly higher than those of SC and SI ($P < 0.01$). The NDF content was lowest in HI (564 g·kg⁻¹ DM) among the treatments ($P < 0.05$). No significant difference was noted in the EE, WSC, and GE content among the treatments.

3.3. Digestibility, Nutritive Value, and Feed Intake of Silage

The digestibility, nutritive value, and feed intake of silage are shown in **Table 3**. The DM and OM digestibility of HI were the highest (0.721 and 0.723, respectively) among the four treatments ($P < 0.05$). The CP digestibility of HC (0.763) and HI (0.821) were higher than those of SC (0.722) and SI (0.729) ($P < 0.01$). The EE digestibility of SI (0.743) was significantly higher than that of SC (0.668) ($P < 0.05$), and that of HI (0.737) was significantly higher than that of HC (0.704) ($P < 0.05$). The ADF and NDF digestibility of HI were high and those of SC were low.

Table 2. Fermentation quality and chemical composition of silages.

Item	S		H		SEM	Significant		
	C	I	C	I		F	I	F × I
Fermentation Quality								
pH	4.75	4.46	4.75	4.51	0.05	NS	**	NS
Lactic Acid (g·kg ⁻¹ DM)	15.9	34.3	24.1	35.3	2.6	**	**	NS
Acetic Acid (g·kg ⁻¹ DM)	35.2	24.7	36.9	25.6	3.3	NS	*	NS
Propionic Acid (g·kg ⁻¹ DM)	1.0	1.4	0.5	0.8	0.1	**	**	NS
Butyric Acid (g·kg ⁻¹ DM)	0.1	0.1	0.3	0.2	0.1	NS	NS	NS
NH ₃ -N (g·kg ⁻¹ TN)	124.6	83.9	105.5	82.7	5.4	*	**	*
V-Score	77.8	91.9	85.1	92.0	1.8	**	**	**
Chemical Composition								
DM (g·kg ⁻¹)	135	138	134	137	18	NS	NS	NS
OM (g·kg ⁻¹ DM)	901	908	904	907	13	NS	**	NS
CP (g·kg ⁻¹ DM)	138	134	171	165	39	**	NS	NS
EE (g·kg ⁻¹ DM)	54	55	56	57	2	NS	NS	NS
ADF (g·kg ⁻¹ DM)	384	390	373	358	3	**	NS	*
NDF (g·kg ⁻¹ DM)	612	594	586	564	8	*	*	NS
WSC (g·kg ⁻¹ DM)	17	17	17	18	1	NS	NS	NS
GE (MJ·kg ⁻¹ DM)	21.5	20.9	21.3	21.2	0.1	NS	NS	NS

S: standard level nitrogen fertilizer rate; H: high level nitrogen fertilizer rate; C: non-inoculant; I: inoculant; SEM: standard error of the mean; F: fertilizer rate; NS: not significant; *: $P < 0.05$; **: $P < 0.01$; NH₃-N: ammonia nitrogen; TN: total nitrogen; V-score (X): $X > 80$, good; $60 < X \leq 80$, middle; $X \leq 60$, poor; DM: dry matter; OM: organic matter; CP: crude protein; EE: ether extract; ADF: acid detergent fiber; NDF: neutral detergent fiber; WSC: water soluble-carbohydrate; GE: gross energy.

Table 3. Digestibility, nutritive value and feed intake of silages.

Item	S		H		SEM	Significant		
	C	I	C	I		F	I	F × I
Digestibility								
DM	0.634	0.676	0.664	0.721	0.013	NS	*	NS
OM	0.636	0.679	0.665	0.723	0.013	NS	*	NS
CP	0.722	0.729	0.763	0.821	0.013	**	NS	NS
EE	0.668	0.743	0.704	0.737	0.012	NS	*	NS
ADF	0.622	0.662	0.639	0.696	0.014	NS	NS	NS
NDF	0.645	0.668	0.659	0.709	0.013	NS	NS	NS
GE	0.621	0.667	0.656	0.710	0.023	NS	NS	NS
Nutritive Value								
DCP (g·kg ⁻¹ DM)	99.6	97.4	130.1	135.1	4.6	**	NS	NS
TDN (g·kg ⁻¹ DM)	618	668	651	709	13		NS	*
DE (MJ·kg ⁻¹ DM)	13.3	13.9	14.0	15.1	0.3	NS	NS	NS
Feed Intake								
DM (g·kg ^{-0.75} day ⁻¹)	34.9	32.5	33.0	35.7	0.7	NS	NS	NS
DCP (g·kg ^{-0.75} day ⁻¹)	3.5	3.2	4.3	4.8	0.2	**	NS	NS
TDN (g·kg ^{-0.75} day ⁻¹)	21.6	21.7	21.5	25.2	0.7	NS	NS	NS
DE (MJ·kg ^{-0.75} day ⁻¹)	0.46	0.45	0.46	0.53	0.02	NS	NS	NS

S: standard level nitrogen fertilizer rate; H: high level nitrogen fertilizer rate; C: non-inoculant; I: inoculant; SEM: standard error of the mean; F: fertilizer rate; NS: not significant; *: $P < 0.05$; **: $P < 0.01$; DM: dry matter; OM: organic matter; CP: crude protein; EE: ether extract; ADF: acid detergent fiber; NDF: neutral detergent fiber; GE: gross energy; DCP: digestible crude protein; TDN: total digestible nutrient; DE: digestible energy.

The DCP contents of HC (130.1 g·kg⁻¹ DM) and HI (135.1 g·kg⁻¹ DM) were significantly higher than those of SC (99.6 g·kg⁻¹ DM) and SI (97.4 g·kg⁻¹ DM) ($P < 0.01$). No significant difference was noted between HC and HI. The TDN content of SC (618 g·kg⁻¹ DM) was the lowest ($P < 0.05$), and that of HI (709 g·kg⁻¹ DM) was the highest ($P < 0.05$). The DE content of SC (13.3 MJ·kg⁻¹ DM) was the lowest and that of HI (15.1 MJ·kg⁻¹ DM) tended to be high. No significant difference was noted in the DM, TDN, and DE intake among the four treatments, but that in the HI-fed treatment was the highest. The DCP intakes of HC (4.3 g·kg⁻¹ DM) and HI (4.8 g·kg⁻¹ DM) were significantly higher than those of SC (3.5 g·kg⁻¹ DM) and SI (3.2 g·kg⁻¹ DM) ($P < 0.01$).

4. Discussion

The CP content of grass material H was high, as previously reported [8] [24], suggesting that high-N fertilization promoted N metabolism of the plant, and protein was synthesized from absorbed N and carbohydrate produced by photosynthesis [25]. The low WSC content of grass material H may have been due to promotion of vegetative growth by high-N fertilization, which increased the yield consuming sugars [14] [26]. The glucose, fructose, and sucrose contents were not markedly different between grass materials S and H, suggesting that the N fertilizer rate did not have a marked influence on mono- and disaccharides. Accordingly, the decrease in the WSC content of the grass materials was assumed to be due to a decrease in the fructosan content, which is a polysaccharide [26].

The pH of HC was 4.2 or higher and the NH₃-N ratio was 100 g·kg⁻¹ DM or higher, showing poor fermentation. The fermentation of SC was also poor, being only slightly different from that of HC. It is unlikely that only the high CP content was involved in the poor fermentation of HC. The fermentation of silage of high-N-fertilized grass was described as being poor in many reports [13] [27] [28]. The fermentation depends on pH elevation, reduction of the lactic acid content, and an increase in the NH₃-N content, and the causes of these include high CP content of grass materials and degradation products of fermentation [29] [30] and low WSC content of grass materials [31]. On the basis of the findings of our study, there may be another factor influencing fermentation other than high CP content and low WSC content.

Lactic acid fermentation was promoted and the NH₃-N ratio decreased in HI compared with those in HC, which improved the fermentation. The addition of an inoculant improved the fermentation of grass silage in many studies [32]-[35]. A marked improvement was also noted in SI, strongly suggesting that the influence of the inoculant was exhibited because the number of lactic acid bacteria adhering to the grass material was markedly low. A marked effect of inoculant was frequently noted when the WSC content of the grass material was 50 - 60 g·kg⁻¹ DM or higher [36] [37], and this may be another factor causing the effect.

The CP content differed by 33 g·kg⁻¹ DM between SC and HC, and the CP digestibility of HC tended to be higher. Regarding the chemical composition and digestibility of high-N-fertilized silage, Izumi *et al.* [27] reported that, when the N fertilizer rate was set to the range of 30 - 90 kg·ha⁻¹, the CP content of timothy silage increased from 143 to 173 g·kg⁻¹ DM with an increase in the N fertilizer rate, and the CP digestibility improved from 0.681 to 0.768. Poulton & Woelfel also [2] reported similar findings, suggesting that changes in the CP digestibility with an increase in N fertilizer rate are influenced by the CP content. In contrast, the CP digestibility of HI was not significantly different from that of HC, and this was similar to findings of the addition of inoculant to timothy silage reported by Souma *et al.* [38], alfalfa silage reported by Cao *et al.* [39], and corn silage reported by Aksu *et al.* [40], showing that inoculant has only a small influence on CP digestibility.

The DCP content and DCP intake of HC were higher than those of SC, and the DCP intake of the sheep increased upon feeding on HC. However, the differences in the TDN and DE contents, and DM, TDN and DE intakes were small. Regarding the DCP and TDN contents, and DM, DCP and TDN intakes of high-N-fertilized silage, Poulton & Woelfel [2] and Izumi *et al.* [27] [41] reported that the DCP content and the DCP intake of silage increased with an increase in the N fertilizer rate, but no influence was noted on the TDN content and TDN intake, and Ataku & Narasaki [42] reported that an increase in the N fertilizer rate did not influence the DM and TDN intakes of the silage, similarly to our findings. However, the TDN content increased in HI compared with that in HC, showing the effect of the inoculant. The improvement of CP and EE digestibility may have been associated with the increase in TDN content. No difference was noted in the DM and TDN intake between HI and HC, which may have been due to the absence of an increase in the DM intake of HI, not leading to an increase in the TDN intake, although the TDN content increased. Regarding the DM intake of silage with the addition of

inoculant, Winters *et al.* [43] reported an increase in the intake of Italian ryegrass silage, whereas no increases in the intake of perennial ryegrass, alfalfa, and mixed-seeded grass and legume silage were reported by Patterson *et al.* [44], Kung *et al.* [45], and Stokes [46], respectively, showing no consistent tendency. Since the DCP and TDN contents, and DCP intake of HI were higher than those of SC and SI, silage with the high DCP and TDN contents, and DCP intake may have been prepared by the addition of inoculant to the high-CP grass material.

5. Conclusion

The high-N fertilizer rate increased the CP content of the grass material by 38 g·kg⁻¹ DM, and the CP content of silage (HC and HI) also increased. The CP digestibility was high in sheep fed HC and HI, and the DCP content and DCP intake increased. The addition of the inoculant improved the silage fermentation and increased the TDN content. On the basis of these findings, silage with high CP content could be prepared by increasing the N fertilizer rate, and high fermentation could be achieved by the addition of the inoculant, which increased the DCP intake of sheep.

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