Comparative Study on the Effects of Combined Treatments of Lactic Acid Bacteria and Cellulases on the Fermentation Characteristic and Chemical Composition of Rhodesgrass (*Chloris gayana* Kunth.) and Italian Ryegrass (*Lolium multiflorum* Lam.) Silages

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ABSTRACT : Prior to ensiling Rhodesgrass (RG) and Italian ryegrass (IRG) were treated with lactic acid bacteria (LAB) or with LAB+cellulases to compare their fermentation characteristics and chemical compositions. LAB (*Lactobacillus casei*) was added to all ensiling materials (except the untreated control) of RG and IRG at a concentration of 1.0×10^5 cfu.g^{-1} fresh forage. The enzymes used were Acremoniumcellulase (A), Meicelase (M) or a mixture of both (AM). Each enzyme was applied at levels of 0.005, 0.01 and 0.02 % of fresh forage. The silages with each treatment were incubated at 20, 30 and 40℃ and stored for about 2 months. While no marked differences were found between the RG and IRG silages with various treatments on dry matter (DM), volatile basic nitrogen (VBN) and water soluble carbohydrate (WSC) contents, there were significant differences in pH value, and lactic acid and butyric acid contents. LAB inoculation did not affect the fermentation characteristics of either the RG or IRG silages. The combined treatments of LAB+cellulases improved the fermentation quality of both the RG and IRG silages as evidenced by the decrease in pH value and increase in lactic acid content. Increasing the amount of added cellulase resulted in a decrease in pH value and an increase in lactic acid content in both the RG and IRG silages. Cellulases A and AM had a greater effect than cellulase M on the fermentation quality of the RG and IRG silages. Incubation temperatures of 30 and 40℃ appeared to be more appropriate environments for stimulating good fermentation than 20℃. (*Asian-Aus. J. Anim. Sci. 1999. Vol. 12, No. 4 : 525-530*)

Key Words : Cellulase, Chemical Composition, Fermentation Quality, Italian Ryegrass, Lactic Acid Bacteria, Rhodesgrass

INTRODUCTION

Existing knowledge on silage making and the ensiling process for herbage species of tropical origin has been based on studies of temperate origin herbage species (Catchpoole and Henzel, 1971; Kim and Uchida, 1990). However, tropical and temperate herbage species are different in their chemical, physical and physiological properties, which could cause a difference in the ensiling process as well as the silage quality between these species types (Kim and Uchida, 1990). According to McDonald et al. (1991), in temperate origin grasses, fructants are the most abundant of the water soluble carbohydrates (WSCs), being present in concentrations of about 50 to 90 g.kg^{-1} DM. On the other hand, grasses of tropical and subtropical origins accumulate starches instead of these fructants. Moreover, McDonald et al. (1991) reported that the WSC can be fermented rapidly by lactic acid bacteria (LAB). In contrast, the most naturally occurring LABs do not have the ability to ferment starch directly. Sheperd and Kung, Jr., (1996a,b) reported that when soluble sugars were limited, enzyme additives should improve the fermentation characteristics by releasing soluble sugars used by the LAB during the fermentation process. This results in a lower final pH value and a higher lactic acid content in the silage. Furthermore, enzyme additives may degrade a portion of the plant cell wall during storage and potentially improve animal performance. There have been extensive studies on the effects of addition of cellulase, alone or mixed with microbial inoculant to silage, although with different results. However, very few studies have compared tropical and temperate herbage treated with enzymes, either alone or in combination with microbial inoculant.

The objective of this study was to compare the fermentation characteristics and chemical compositions of silages made from Rhodesgrass (*Chloris gayana* Kunth.) and Italian ryegrass (*Lolium multiflorum* Lam.) treated with lactic acid bacteria (LAB) and/or different types and levels of cellulase, incubated at 20, 30 and 40℃.

MATERIALS AND METHODS

Silage additives

The cellulases and lactic acid bacteria (LAB) used in this experiment were provided by Yukijirushi Syubyo Co. Ltd., Hokkaido, Japan. Two cellulases were used. One was derived from *Acremonium cellulolyticus* (Acremoniumcellulase, cellulase A), and the other was derived from *Tricoderma viride* (Meicelase, cellulase M). In addition, a mixture of these two cellulases (cellulase AM) was used at an A:M ratio of 1:2. The inoculant LAB (Snow Lact-L) was guaranteed by the manufacturer to contain a minimum of 2.5×10^{10} cfu.g^{-1} powder of *Lactobacillus casei*. Each cellulase preparation was applied at levels of 0.005, 0.01 and 0.02 % (fresh matter). The inoculant LAB was used at a theoretical

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application rate of 1.0×10^5 cfu.g^{-1} fresh sample forage. On the day of making silages, a certain amount of each cellulase preparation or inoculant LAB was diluted in distilled water to achieve the required concentration, and kept for silage making.

Silage making

The first growth of Rhodesgrass (RG) was harvested at the heading stage with a hand cutter on 9 August 1995. This harvested material was chopped into approximately 1.3 cm lengths and then lacerated with a chopper-cracker (Taninaka Co. Ltd.). One ml of the LAB inoculant solution with or without 1 ml cellulase solution was sprayed onto 1 kg of grass sample with a 2.5-ml syringe. The sample was then thoroughly mixed and ensiled into a 2-L vinyl bottle silo. The silage additive treatments were as follows:

Treatment Silage additive

1. Untreated (Control, CTL)
2. LAB (application rate 1.0×10^5 cfu.g^{-1} fresh sample)
3. LAB+A 0.005 %
4. LAB+A 0.01 %
5. LAB+A 0.02 %
6. LAB+M 0.005 %
7. LAB+M 0.01 %
8. LAB+M 0.02 %
9. LAB+AM 0.005 %
10. LAB+AM 0.01 %
11. LAB+AM 0.02 %

Nine silages were made for each treatment (a total of 99 silages). Three silages for each treatment were incubated at 20, 30 and 40℃ for approximately 2-months storage period.

On 9 May 1996, the first growth of Italian ryegrass (IRG) was harvested at the heading stage and used for silage making with the same procedures and treatments applied to Rhodesgrass material described above. The chemical composition of the experimental forages prior to ensiling is presented in table 1.

Table 1. Chemical compositions of Rhodesgrass and Italian ryegrass materials prior to ensiling

Content	Rhodesgrass	Italian ryegrass
Dry matter (%)	21.76	21.76
Crude ash (% DM)	10.28	12.22
Organic matter (% DM)	89.72	87.78
Crude protein (% DM)	10.38	18.03
NDF (% DM)	66.42	59.22
ADF (% DM)	38.18	32.13
Hemicellulose (% DM)	28.24	27.09
Cellulose (% DM)	31.04	28.15
ADL (% DM)	7.14	3.98
WSC (% DM)	5.01	7.17

Abbreviated : NDF = Neutral detergent fibre, ADF = Acid detergent fibre, ADL = Acid detergent lignin, WSC = Water soluble carbohydrate, Hemicellulose = NDF - ADF, Cellulose = ADF - ADL.

After the incubation period, the silos were opened and the upper 1/5 of each silage was discarded before sampling. The samples were collected and stored at -32℃ until they were used for further analysis.

Chemical analysis

Dry matter contents of material grasses and silages were determined by a vacuum freeze-dry method (Uchida, 1986). The dried samples were ground and then crude protein was determined by the Kjeldahl method and WSC was evaluated by using the method of Deriaz (1961).

Water soluble extracts were prepared by macerating of 40 g fresh sample silage in 400 ml distilled water. The pH of the extracts was measured by a Horiba F-12 pH meter, organic acid and ethanol were measured by gas chromatography (GC-14A, Shimadzu) as described by Uchida and Hayashi (1985), lactic acid was analyzed by the method of Barker and Summerson (1941), and volatile basic nitrogen (VBN) was measured by steam distillation method.

Statistical analysis

A two-sample T-test was applied to all data. For comparisons of the additive treatments, data for all incubation temperatures were combined. Conversely, for comparisons of incubation temperatures, data for all additive treatments were combined.

RESULTS AND DISCUSSION

Silage quality

The differences in pH values and lactic acid concentrations between the RG and IRG silages in all treatments are shown in figures 1 and 2, respectively. The organic acids, ethanol and the other chemical components are shown in tables 2 and 3.

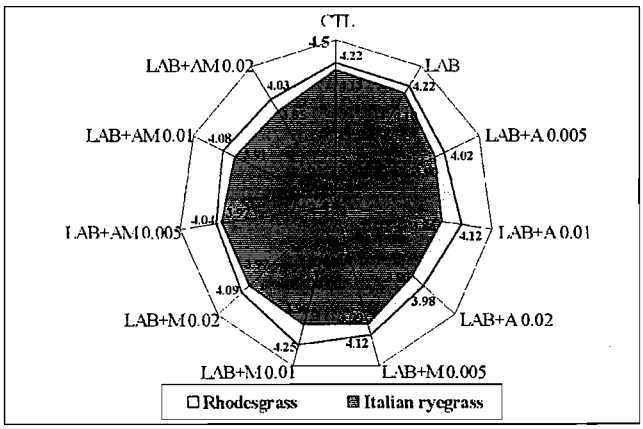

Figure 1. Differences in pH values between RG and IRG silages made with various treatments. These pH values were significantly different ($p<0.01$) between the two species

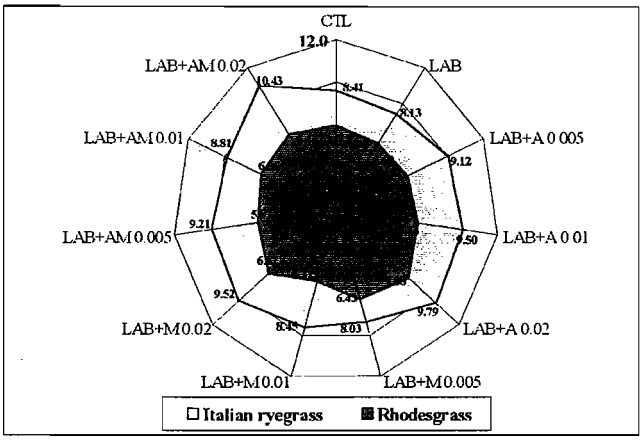

Figure 2. Differences in lactic acid contents (% DM) between RG and IRG silages made with various treatments. These percentages were significantly different ($p<0.01$) between the two species

Both of the RG and IRG silages were well preserved as evidenced by their low pH values (4.02-4.22 in RG and 3.80-4.13 in IRG silages), high lactic acid concentrations (5.11-7.08 in RG and 8.03-10.43 in IRG silages, % DM), and low butyric acid concentrations (0.11-0.27 in RG and 0.02-0.08 in IRG silages, % DM), regardless of the treatment and incubation temperature.

Propionic acid was present at low levels in the RG silages, but was not detectable in most of the IRG silages (except the untreated control). Good fermentation in these silages was also shown by low VBN concentrations (1.86-2.69 in RG and 1.98-2.45 in IRG silages, % total nitrogen/TN). These values were lower than the ammonia-N content suggested by Henderson (1993) that a well preserved silage should have less then 8 % TN. The fact that both the RG and IRG silages were well preserved may indicate that the WSC contents in the original grasses (5.10 % DM for RG

Table 2. Differences in organic acids and ethanol contents between RG and IRG silages made with various treatments

Treatment	Acetic acid (% DM)			Propionic acid (% DM)			Butyric acid (% DM)			Ethanol (% DM)		
	RG	IRG	Sig[1]	RG	IRG	Sig[1]	RG	IRG	Sig[1]	RG	IRG	Sig[1]
Control	1.35	1.19	NS	0.08	0.02	**	0.16	0.08	**	0.40	0.97	**
LAB	1.32	1.17	NS	0.05	ND	-	0.13	0.04	**	0.43	1.01	**
LAB+0.005 A	1.19	1.32	NS	0.03	ND	-	0.12	0.03	**	0.49	0.96	**
LAB+0.01 A	1.06	1.27	NS	0.03	ND	-	0.16	0.02	**	0.65	1.01	*
LAB+0.02 A	1.22	1.27	NS	0.01	ND	-	0.12	0.02	**	0.59	0.95	*
LAB+0.005 M	1.26	1.18	NS	0.04	ND	-	0.12	0.02	**	0.46	0.93	**
LAB+0.01 M	1.28	1.15	NS	0.03	ND	-	0.14	0.05	**	0.50	1.02	**
LAB+0.02 M	1.27	1.09	NS	0.03	ND	-	0.15	0.03	**	0.70	0.93	NS
LAB+0.005 AM	1.19	1.22	NS	0.01	ND	-	0.11	0.02	**	0.42	1.06	**
LAB+0.01 AM	1.37	1.19	NS	0.02	ND	-	0.27	0.03	**	0.83	1.01	NS
LAB+0.02 AM	1.14	1.76	**	0.02	ND	-	0.19	0.02	**	0.68	1.00	**

Abbreviated: ND = Not detectable. [1] Significant differences : NS $p>0.05$, * $p<0.05$, ** $p<0.01$.

Table 3. Differences in dry matter, total N, VBN and WSC contents between RG and IRG silages made with various treatments

Treatment	Dry matter (DM)			Total N (% DM)			VBN (% TN)			WSC (% DM)		
	RG	IRG	Sig[1]	RG	IRG	Sig[1]	RG	IRG	Sig[1]	RG	IRG	Sig[1]
Control	21.98	22.81	NS	1.61	2.81	**	2.66	2.45	NS	1.02	1.02	NS
LAB	22.35	22.58	NS	1.61	2.85	**	2.20	2.04	NS	0.96	1.03	NS
LAB+0.005 A	22.22	22.38	NS	1.57	2.85	**	1.86	1.99	NS	1.33	1.36	NS
LAB+0.01 A	21.92	22.27	NS	1.64	2.84	**	2.24	2.05	NS	1.12	1.57	**
LAB+0.02 A	22.05	22.20	NS	1.64	2.86	**	2.36	1.98	*	1.56	1.74	NS
LAB+0.005 M	21.94	22.74	NS	1.61	2.81	**	2.23	2.11	NS	1.22	1.30	NS
LAB+0.01 M	22.26	22.68	NS	1.62	2.82	**	2.69	2.02	**	0.99	1.31	*
LAB+0.02 M	21.88	22.54	NS	1.63	2.82	**	2.69	2.38	NS	1.37	1.18	NS
LAB+0.005 AM	22.06	22.47	NS	1.63	2.80	**	2.01	2.30	NS	1.35	1.33	NS
LAB+0.01 AM	22.00	22.49	NS	1.66	2.81	**	2.40	2.08	NS	1.10	1.42	*
LAB+0.02 AM	22.05	22.54	NS	1.64	2.86	**	2.34	2.26	NS	1.29	1.49	NS

Abbreviated : VBN = Volatile basic nitrogen, TN = Total nitrogen, WSC = Water soluble carbohydrate.
1) see table 2.

and 7.17 % DM for IRG) were sufficient as substrates for lactic acid bacteria (LAB) for producing lactic acid and reducing the pH. The critical content of WSC needed to obtain a satisfactory silage fermentation has been suggested to be somewhere around 2-3 % fresh matter (Selmer-Olsen, 1994) or around 3-5 % dry matter (Weinberg et al., 1995). The good preservation of these silages may also be due to the fact that the dry matter (DM) contents of the original grasses were at an ideal level, since the DM content of both grasses was 21.76 %. McDonald et al. (1991) recommended that the characteristics of an ideal crop for preservation as silage are that it should contain an adequate level of fermentable substrate in the form of water soluble carbohydrate and a dry matter content above 20 %.

Herbage species

The fermentation appeared to be more effective in IRG silages as shown by the lower ($p<0.05$) pH value and higher ($p<0.05$) lactic acid concentration than those in the RG silages, in all treatments regardless of the incubation temperature (figures 1 and 2). The IRG silages also had a lower ($p<0.01$) butyric acid concentration and had almost no propionic acid (except in the untreated control silage) compared with the RG silages, but there was no difference in the acetic acid concentration between the two silages. The differences in the fermentation characteristics between the RG and IRG silages might be caused by the higher ($p<0.01$) WSC content in IRG than in RG (table 1). According to McDonald et al. (1991) the WSC contents of tropical grasses are generally lower than those of temperate grasses. Moreover, since the tropical and temperate herbage species are different in their chemical, physical and physiological properties, these differences might result in differences in the silage quality between tropical and temperate species (Kim and Uchida, 1990).

The VBN concentration was low (less than 3 % TN) in both the RG and IRG silages. The VBN concentration tended to be lower in the IRG silages than in RG silages, but statistically there were almost no difference between the two silages in all treatments, except that in the LAB+0.02 A and LAB+0.01 M treatments. This might indicate that both the RG and IRG grasses have the ideal chemical compositions (more than 5% WSC content and above 20% dry matter content) to sustain good fermentation. The VBN concentration was lower ($p<0.05$) in the treated silages than in the untreated control. This suggest that treatments with LAB and LAB+cellulases caused the pH value to quickly fall, which, in turn, partially inhibited plant proteolysis and possibly clostridial deamination of amino acids (Kung, Jr., et al., 1990). The tendency of the IRG silages to have a low VBN concentration might indicate that fermentation was more effective in the IRG silages than in the RG silages.

The residual WSC content was almost the same in both the RG and IRG silages, except that the WSC contents in the LAB+0.01 A, the LAB+0.01 M and the LAB+0.01 AM treatments were higher ($p<0.05$) in the IRG silages than in the RG silages. For both the RG and IRG silages, residual WSC contents were higher with the combined treatments of LAB+cellulases than with either the LAB-treatment or no treatment. This might be because the degradation of the carbohydrate fraction by the addition of the cellulases provided greater WSC contents in both the RG and IRG silages. According to Sheperd et al. (1995), the increase in the glucose content of the cellulase-treated silages might indicate that the added enzymes continued to hydrolyze substrate even after the fermentation was complete, but large amounts of residual WSC could lead to aerobic instability of the silage.

The present results were not in agreement with the data of Kim and Uchida (1990) who reported that the low lactic acid concentration and high acetic acid and VBN concentrations in the RG silages compared with those in the IRG silages as might be due to the low WSC (4.14 % DM for RG and 14.69 % DM for IRG) content in RG material. In particular, these findings showed that the fermentation of silages of tropical origin could be as good as those of temperate origin if the grass prior to ensiling contains an ideal level of dry matter and sufficient substrate.

LAB inoculation

The fermentation quality and the chemical composition of the untreated control and the LAB-treated silages were almost the same for both grasses. Inoculation of LAB only caused a significant decrease ($p<0.05$) in the VBN concentrations in both the RG and IRG silages (table 3). The failure of LAB treatment to improve the quality of both RG and IRG silages might be because the WSC contents in the original grasses (5.10% DM for RG and 7.17% DM for IRG) could not allow the LAB to produce enough lactic acid to further reduce the final pH value (Ridla and Uchida, 1998a,b). These results were in line with the findings of Tamada et al. (1996) in napier grass silage, who reported that LAB inoculation did not affect on lowering pH value and increasing lactic acid concentration due to the low WSC content in the original grass (4.22 % DM). Similar results were reported by Keady and Murphy (1996) in silages from ryegrass swards. They found that inoculant treatment did not alter the silage fermentation relative to untreated silage, as the WSC values were 1.82% and 1.66 % fresh matter for untreated and inoculant treated, respectively.

Cellulase addition

The combined treatments of LAB+cellulases improved the fermentation quality of both the RG and IRG silages as evidenced by their lower ($p<0.01$) pH value and higher ($p<0.05$) lactic acid concentration compared with those of both the untreated control and LAB-treated silages (figures 1, 2 and table 3). These treatments had no effect on the ethanol, acetic acid, butyric acid or VBN concentrations (tables 2 and 3). Increasing the

Table 4. Differences in fermentation characteristics and chemical compositions between RG and IRG silages at different incubation temperatures

	20℃			30℃			40℃		
	RG	IRG	Sig[4]	RG	IRG	Sig[4]	RG	IRG	Sig[4]
pH	4.12	3.96	**	4.09	3.93	**	4.09	3.97	**
Dry matter (%)	22.36	22.76	NS	21.72	22.19	NS	22.62	22.18	NS
Ethanol (% DM)	0.42	1.31	**	0.39	0.88	**	0.81	0.77	NS
Lactic acid (% DM)	5.68	8.51	**	5.11	10.44	**	8.02	8.54	*
Acetic acid (% DM)	1.40	0.94	**	1.44	1.60	*	1.03	0.99	NS
Propionic acid (% DM)	0.04	ND	-	0.01	ND	-	0.06	ND	-
Butyric acid (% DM)	0.22	0.03	**	0.20	0.06	**	0.31	0.04	**
TN[1] (% DM)	1.56	2.91	**	1.72	2.72	**	1.57	2.78	**
VBN[2] (% TN)	2.28	1.82	**	2.32	1.94	**	2.60	2.60	NS
WSC[3] (% DM)	1.26	1.13	NS	1.08	1.24	*	1.25	1.71	**

[1, 2, 3] See table 3; [4] see table 2.

amount of cellulase resulted in a decrease ($p<0.01$) in the pH value and an increase ($p<0.01$) in the lactic acid concentration of both the RG and IRG silages, in all of cellulase types, regardless of incubation temperature. These data showed that cellulase addition improved the preservation of silages by decreasing the pH value and increasing lactic acid concentration, which were consistent with our previous findings (Ridla and Uchida, 1993; Ridla and Uchida, 1997) and those of other published studies (Henderson and McDonald, 1977; Jacobs et al., 1991; Selmer-Olsen et al., 1993; Sheperd et al., 1995; van Vuuren et al., 1989). This might be due to a greater amount of fermentable carbohydrates (WSC) provided by the hydrolysis of cell wall components, which stimulate fermentation by lactic acid bacteria (Ridla and Uchida, 1998a,b). The residual WSC contents of the silages receiving the combined treatments (LAB+cellulases) were higher ($p<0.01$) than those of both the untreated control and LAB-treated silages. Increasing the amount of added cellulase caused a significant increase ($p<0.05$) in the residual WSC content of all cellulase types. This might indicate that the reduction of cell wall components by cellulase was able to provide more WSC, which would help to sustain fermentation by LAB in the silo (Ridla and Uchida, 1998a,b). This finding was consistent with the results of Jaakkola (1990), Jacobs and McAllan (1992), Jacobs et al. (1992), McDonald et al. (1991), Ridla and Uchida (1993), Ridla and Uchida (1997), Sheperd et al. (1995) and Stokes (1992) who reported that enzyme addition was capable of breaking down the components of structural carbohydrates in the silo and provided more WSC as substrate for the silage fermentation.

Cellulase type

In both the RG and IRG silages, fermentation quality was the best when the silages were treated with cellulase A, and was nearly as good as when the silages were treated with cellulase AM. On the other hand, silages treated with cellulase M resulted in much poorer fermentation. The data showed that the silages treated with cellulases A and AM had lower ($p<0.01$) pH values and higher ($p<0.05$) lactic acid concentrations than those of silages treated with cellulase M. The silages treated with cellulases A and AM also had higher ($p<0.01$) residual WSC concentrations compared with silages treated with cellulase M (Ridla and Uchida, 1998a,b). This was in line with the data reported by Tomoda et al. (1996) in which alfalfa silage treated with a cellulase preparation originating from *Acremonium cellulolyticus* resulted in a lower pH and a higher lactic acid concentration than was obtained with a cellulase preparation originating from *Tricoderma viride*. In addition, this result was consistent with the results of Zhang et al. (1997a,b) who found that treatment of straw silages with Acremonium cellulase resulted in better fermentation quality than did treatment with Meicelase, and that treatment with a mixture of these cellulases had an intermediate effect.

Incubation temperature

Incubation temperatures of 30℃ and 40℃ seemed to be more effective for the fermentation of the IRG and RG silages, respectively, and these temperatures might be independent of the additive treatments (table 4). The data showed that, at the above temperatures, the pH value was lower ($p<0.05$) and lactic acid concentration was higher ($p<0.01$) than they were at other incubation temperatures.

In conclusion, fermentation more effectively occurred with Italian ryegrass silages than with Rhodesgrass silages. The chemical, physical and physiological differences between the two grasses may lead to different fermentation characteristics. As with silage made from temperate origin herbage species, good preservation in silage made from tropical origin herbage species could be achieved with the ideal dry matter content and a sufficient amount of fermentable substrate. Fermentation quality of Rhodesgrass and Italian ryegrass silages was not affected by LAB inoculation, but it was improved by the combined treatment with LAB+ cellulases.

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