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Development of a new lactic acid bacterial inoculant for fresh rice straw silage

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Objective: Effects of newly isolated *Lactobacillus plantarum* on the fermentation and chemical composition of fresh rice straw silage was evaluated in this study.
Methods: Lactic acid bacteria (LAB) from good crop silage were screened by growing them in MRS broth and a minimal medium with low carbohydrate content. Selected LAB (LAB 1821) were Gram-positive, rods, catalase negative, and were identified to be *Lactobacillus plantarum* based on their biochemical characteristics and a 16S rRNA analysis. Fresh rice straw was ensiled with two isolated LAB (1821 and 1841), two commercial inoculants (HM/F and P1132) and no additive as a control.
Results: After 2 months of storage at ambient temperature, rice straw silages treated with additives were well-preserved, the pH values and butyric and acetic acid contents were lower, and the lactic acid content and lactic/acetic acid ratio were higher than those in the control ($p<0.05$). Acidity (pH) was lowest, and lactic acid highest, in 1821-treated silage ($p<0.05$). The NH_3-N content decreased significantly in inoculant-treated silage ($p<0.05$) and the NH_3-N content in 1821-treated silage was lowest among the treatments. The dry matter (DM) content of the control silage was lower than that of fresh rice straw ($p<0.05$), while that of the 1841- and p1174-inoculant-treated silages was significantly higher than that of HM/F-treated silage. Microbial additives did not have any significant ($p>0.05$) effect on acid detergent fiber or neutral detergent fiber contents. Crude protein (CP) content and *in vitro* DM digestibility (IVDMD) increased after inoculation of LAB 1821 ($p<0.05$).
Conclusion: LAB 1821 increased the CP, IVDMD, lactic acid content and ratio of lactic acid to acetic acid in rice straw silage and decreased the pH, acetic acid, NH_3-N, and butyric acid contents. Therefore, adding LAB 1821 improved the fermentation quality and feed value of rice straw silage.

Keywords: Rice Straw Silage, Inoculant, Lactic Acid Bacteria, Fermentation, Quality

INTRODUCTION

The annual roughage demands of herbivorous animals in Korea are estimated to be about 5.6 million tons. Roughage self-sufficiency is about 82% with half of them being rice straw. More than 2 million tons of rice straw is typically used to raise Korean native cattle [1]. However, rice straw is of low quality and does not provide enough nutrients for most ruminant animals. More than half of the dry matter (DM) of rice straw consists of cellulose and hemicellulose, and the remainder is comprised of lignin, nitrogenous compounds, and ash [2]. The feed value of rice straw as hay is very poor because of its structural characteristics and long drying period. Although rice straw contains sufficient cellulose to make it an excellent energy source for ruminants, it is a poor-quality feed in its natural state.

As a means of improving rice straw quality various agents were used in the past and one of them was ammonia, which increases crude protein (CP) content. The palatability of rice straw

for ruminants is low but this low palatability can be increased by cubing or pelleting the straw [3]. Wrapping large round bales with stretch-wrap plastic film to make silage is a relatively new method of preserving forage. However, it is difficult to make good silage from fresh rice straw due to low water soluble carbohydrate (WSC) content at harvest, hollow stems, and small amount of epiphytic lactic acid bacteria (LAB) [4].

Inoculants are the most common biological additives used to preserve silage worldwide. These products have selected strains of homofermentative LAB, such as *Lactobacillus plantarum*, *Enterococcus faecium*, and *Pediococcus* spp. [5]. Adding LAB helps decrease pH faster, lower the final pH value, increase lactic acid content, and decrease NH_3-N in silage [6]. Many microbial inoculant products are available commercially but most of them were not developed for rice straw but rather for major foraging crops, such as corn, sorghum, alfalfa, and grasses. Therefore, we tried to isolate, identify and evaluate novel LAB strains suitable for low WSC content rice straw silage.

MATERIALS AND METHODS

Collection and screening of microbes

Silage samples without any additives were collected from around the country. The samples were plated on MRS agar and incubated at 35°C for 48 h. LAB were quantified by manually counting all yellow colonies, which were subcultured in MRS broth (Difco, Detroit, MI, USA) to test their growing ability. Among numerous bacteria, 10 microbes were tested for their acid-producing ability in MRS broth for 8 h at 25°C. Finally, two LAB were isolated from the samples (LAB 1821 and 1841).

Silage preparation

Fresh rice straw (Chucheong) was directly ensiled after harvesting on an experimental field at the National Institute of Animal Science of RDA (Cheonan, Korea). The straw was chopped into 2 to 3 cm pieces using a rice straw cutter and mixed well. Then, it was ensiled in an experimental silo (20 L) with or without microbial additives (1821, 1841, HM/F, Agri-Lloyd International Ltd., Leominster, UK; and P1132, Pioneer Hi-Bred International Inc., Plymouth, IN, USA) and stored at ambient temperature (<25°C) for 60 days. The addition rate of inoculant was 1×10^6 cfu/g fresh matter. The control received the same amount of distilled water. Three replications for each treatment were ensiled, and the experimental silo was sealed.

Silage analysis

The silo was opened after 2 months, and the DM contents of the rice straw silages were oven-dried at 65°C for 72 h. The dried samples were ground to pass through a 1 mm screen and kept in double-plug-type plastic bottles for analysis. CP was determined by the Kjeldahl method [7], and acid detergent fiber (ADF) and neutral detergent fiber (NDF) were measured by the method of Goering and Van Soest [8]. The *in vitro* dry matter digestibility (IVDMD) of the rice straw silages was determined using the two-stage technique of Tilley and Terry [9] over 72 h. Ten grams sample of each silage was macerated with 90 mL of distilled water for 30 min in a shaker and filtered, and the filtrates were used to measure pH with a pH meter (HI 9024; Hanna Instruments Ltd., Leighton Buzzard, UK). Ten grams sample of each silage was macerated with 90 mL of distilled water for 24 h and filtered through filter paper (#6). The filtrates were analyzed for volatile fatty acid and lactic acid contents. Volatile fatty acids were analyzed using gas chromatography (Model 3400; Varian Co., Harbor City, CA, USA), and lactic acid was analyzed by high performance liquid chromatography (HP-1100; Hewlett-Packard Co., Palo Alto, CA, USA). WSC was determined using the anthrone method of Thomas [10] and NH_3-N concentrations were analyzed by the method of Chaney and Marbach [11] using a spectrophotometer (UVIDEC-610; Jasco Co., Tokyo, Japan). LAB counts in silage were estimated on MRS agar, and fungal counts were estimated on potato dextrose agar, as described by the American Public Health Association [12].

Lactic acid bacteria isolation

LAB 1821 was isolated from a good oat silage by plating on MRS agar containing 0.02% sodium azide and was identified based on its biochemical characteristics and a 16S rRNA analysis. The bacteria were screened by growing and acid-producing ability on minimal medium with a low carbohydrate content (Table 1). The 16S rRNA gene sequence analysis was performed using the method of Pavlova et al [13].

Statistical analysis

All data were analyzed using the general linear model procedure in SAS software [14]. Differences among treatment means were determined using the least significant difference (LSD) test. A probability level of $p<0.05$ was considered to be statistically significant.

RESULTS

Selection of microbes

The acid-producing ability (pH) of 10 isolated bacteria is shown

Table 1. Low carbohydrate minimal medium used for the screening

Ingredients	Amount
K_2HPO_4 (g)	1.0
KH_2PO_4 (g)	1.0
Yeast extract (g)	5.0
$FeSO_4$ (g)	0.001
$MgSO_4$ (g)	0.25
NaCl (g)	0.005
Glucose (g)	1
Water (mL)	1,000
pH	7.0

Table 2. Change of acid producing ability (pH) for the selected cultures during the incubation

Incubation time (h)	1808	1819	1821	1823	1827	1828	1830	1834	1840	1841	Mean
0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	-
8	4.41^{bc}	4.38^{cde}	4.36^{def}	4.42^{bc}	4.46^{a}	4.43^{abc}	4.41^{bcd}	4.44^{ab}	4.43^{abc}	4.35^{df}	4.41

$^{a-f}$ Means within rows followed different superscript letters are statistically different ($p < 0.05$).

in Table 2. The LAB 1821 and 1841 microbial cultures showed the lowest pH values after an 8 h incubation and both were incubated in MRS broth at 25°C, 35°C, and 45°C for 12 h to evaluate their growth characteristics. The cultures grew well at 25°C and 35°C, but not at 45°C (Figure 1). Finally, strains 1821 and 1841 were selected as new silage inoculant candidates. The isolated strains were Gram-positive, rods, catalase-negative, and were identified to be the non-gas-forming *Lactobacillus plantarum* (*L. plantarum*) (Table 3), based on their biochemical characteristics and substrate utilization. The growing ability of *L. plantarum* 1821 on minimal medium with a low carbohydrate content was not different from that of *L. plantarum* (ATCC 10012).

Chemical composition of fresh rice straw

The chemical composition of fresh rice straw is shown in Table 4. The fresh rice straw had low WSC and CP contents, as well as low IVDMD. Insufficient WSC content could result in poor LAB fermentation during ensiling. The DM, CP, and WSC contents of fresh rice straw were 36.1%, 35.1 g/kg, and 43.3 g/kg, respectively. The ADF and NDF contents, as well as the IVDMD, were 418.9, 657.3, and 417.4 g/kg, respectively.

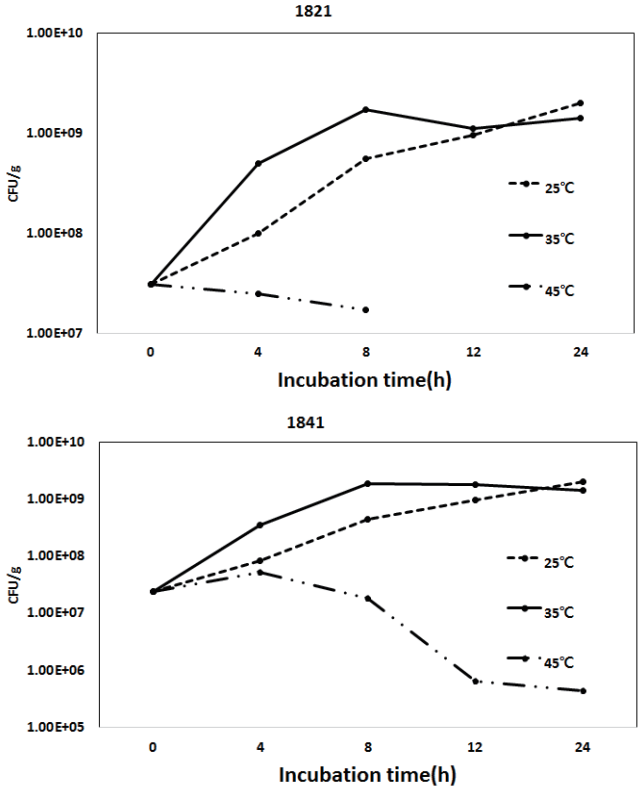

Figure 1. Growth characteristics of selected two microbes (1821; upper, 1841; low) at different temperature.

Effect of the new inoculant on rice straw silage quality

The rice straw silage quality is shown in Table 5. The fresh rice straw silages treated with additives (1821, 1841, HM/F, and P1132) were well-preserved at ambient temperature; the pH value and butyric acid content were lower, while the lactic acid and acetic acid contents, as well as the lactic/acetic acid ratio, were higher than those of the control. Acidity (pH) was lowest and lactic acid highest, in the 1821-treated silage ($p<0.05$). All silages treated with inoculants had higher lactic/acetic acid ratios than the control, of 3.82–9.66. The NH_3-N content decreased significantly in inoculant-treated silage ($p<0.05$), while that of the 1821-treated silage was the lowest among the treatments.

The effect of inoculants on the microbial flora in rice straw silage is shown in Table 4. LAB counts increased in 1821-, 1841-, and P1132-inoculated silages ($p<0.05$), and fungal counts decreased in response to the LAB 1821 treatment ($p<0.05$).

Effect of the new inoculant on rice straw feed value

Table 6 shows the chemical composition of the fresh rice straw

Table 3. The biochemical characteristics of selected lactic acid bacteria, 1821

Item	Characteristics	Major carbohydrate fermentation	+/-
Shape	Rod	L-Arabinose	+
Fermentation type	Homo	Galactose	+
Gram stain	+	Fructose	+
Catalase activity	-	Dulcitol	-
Gas from glucose	-	Sorbitol	+
Growth potential temp. (°C)	13-43	Lactose	+
Growth potential pH	3.5-7.5	Mannitol	+

+, Active; -, Inactive.

Table 4. Nutrient contents and *in vitro* dry matter digestibility of rice straw at ensiling

Composition	Fresh rice straw
DM (%)	36.1
WSC (g/kg DM)	43.3
CP (g/kg DM)	35.1
ADF (g/kg DM)	418.4
NDF (g/kg DM)	657.3
IVDMD (g/kg DM)	417.4

DM, dry matter; WSC, water soluble carbohydrate; CP, crude protein; ADF, acid detergent fiber; NDF, neutral detergent fiber; IVDMD, *in vitro* dry matter digestibility.

Table 5. Effect of inoculant on the acidity (pH), ammonia-nitrogen, organic acid composition and microbial flora in rice straw silage

Items	Control	1821	1841	P1132	HM/F	LSD (p<0.05)
pH	4.93	4.40	4.51	4.73	4.75	0.28
NH_3-N (% TN)	16.83	5.86	6.26	6.07	6.81	0.82
Acetic acid (% DM)	0.67	0.29	0.26	0.50	0.50	0.15
Butyric acid (% DM)	0.35	0.18	0.77	0.14	0.11	0.12
Lactic acid (% DM)	1.80	2.80	2.49	2.65	1.91	0.24
Lactic/acetic acid	2.69	9.66	9.58	5.30	3.82	1.62
LAB (cfu/g)	2.9×10^7	2.1×10^8	1.7×10^8	1.9×10^8	9.1×10^7	4.0×10^7
Fungi (cfu/g)	100	20	150	100	120	30

LSD, least significant difference; TN, total nitrogen; DM, dry matter; LAB, lactic acid bacteria.

Table 6. Effects of inoculant on nutrient contents and *in vitro* dry matter digestibility in rice straw silage

Items	Control	1821	1841	P1132	HM/F	LSD (p<0.05)
DM (%)	30.5	37.6	39.7	40.7	35.0	3.3
WSC (g/kg DM)	9.4	7.2	7.6	7.3	8.2	1.3
CP (g/kg DM)	46.1.	56.0	50.8	52.2	47.5	4.4
ADF (g/kg DM)	491.3	481.7	493.7	484.6	472.8	NS
NDF (g/kg DM)	703.7	712.4	694.2	703.9	696.1	NS
IVDMD (g/kg DM)	333.1	357.6	345.7	367.2	357.4	14.2

LSD, least significant difference; DM, dry matter; WSC, water soluble carbohydrate; CP, crude protein; ADF, acid detergent fiber; NS, not significant; NDF, neutral detergent fiber; IVDMD, *in vitro* dry matter digestibility.

silage. The DM content of the control silage was lower compared with that of fresh rice straw (p<0.05) and all inoculant-treated silages had a significantly higher DM content than that of the HM/F-treated silage. None of the microbial additives had any significant (p>0.05) effect on ADF or NDF contents. The CP content and IVDMD increased after inoculation of the microbes (p<0.05). The WSC content was lower in the inoculant-treated silages by an average of 7.6 g/kg compared to the control (9.4 g/kg).

DISCUSSION

Bacterial inoculants are known to improve silage fermentation and forage conservation. In present study inoculation with LAB significantly reduced pH, as well as the acetic acid, butyric acid and NH_3-N contents, but increased the lactic acid content. Current results imply that LAB inoculation promoted rapid acidification, which inhibited the proteolytic activities of plant enzymes.

Most commercial inoculants consist of LAB. The LAB belong to a group of Gram-positive, low guanine-cytosine (G+C) containing, nonmotile, nonspore-forming, aerotolerant bacteria that ferment hexoses to lactic acid [15]. Microorganisms can be categorized according to their ability to grow at low, moderate, or high temperatures (psychrophilic, mesophilic, and thermophilic microorganisms, respectively) [16]. In general, the proper temperature for good silage fermentation is <32°C [17]. The 1821 and 1841 microbes grew well at 25°C to 35°C in this study, so both were classified as mesophilic. McDonald [18] suggested that an ideal silage inoculant should grow at temperatures of up to 120°F (48°C).

Low WSC content of rice straw in the harvest stage restricts LAB growth during silage fermentation [19]. Therefore, rice straw silage microbes must have good growth characteristics in low WSC content media. LAB 1821 showed better growth ability (pH and viable count) in sugar-restricted media compared with microbes in low WSC content media from the American Type Culture Collection (Table 7), and was identified as *L. plantarum* by the 16s rRNA gene sequence analysis. The 16S rRNA sequence analysis method is highly effective for identifying the genus and species of organisms. LAB 1821 was identified as *L. plantarum* by previous sequencing and phylogenetic tree analyses (Figure 2) [20].

Table 7. Comparison of growth ability of selected LAB in sugar restricted media

Strains	Concentration of sugar (%)	Incubation time					
		0 h		6 h		24 h	
		Viable count (cfu/mL)	pH	Viable count (cfu/mL)	pH	Viable count (cfu/mL)	pH
1821	0.1	5.2×10^7	6.69	5.1×10^8	4.95	4.2×10^8	4.62
	0.5		6.43	6.3×10^8	4.34	7.9×10^8	3.60
Lactobacillus plantarum ATCC 10012	0.1	4.0×10^7	6.70	6.2×10^7	6.16	1.2×10^8	4.65
	0.5		6.43	1.2×10^8	5.00	3.7×10^8	3.87
Lactobacillus bulgaricus ATCC 33409	0.1	4.9×10^7	6.71	2.0×10^8	6.14	4.0×10^8	4.62
	0.5		6.44	2.4×10^8	5.23	3.9×10^8	3.62

LAB, lactic acid bacteria.

Figure 2. Phylogenetic tree of partial 16S rRNA sequence of lactic acid bacteria (LAB) 1821 and identified bacteria in the nucleotide database of GeneBank.

Acidity (pH) is a crucial factor when evaluating silage fermentation quality [21]. Compared with the control silage, the pH of LAB 1821-treated silage decreased significantly ($p<0.05$). All inoculants significantly increased the lactic acid content of rice straw silage, except HM/F. The LAB 1821 and 1842 decreased the acetic acid content.

Adding the LAB decreased the NH_3-N content of rice straw silage significantly ($p<0.05$). McDonald [18] reported that a low pH inhibits protein degradation in silage. In this study, the low pH values in all LAB-treated silages prevented protein degradation. Whiter and Kung [22] stated that homofermentative LAB reduce proteolysis and deamination of silage through a more rapid fall in pH. The absolute value (5.86% to 6.81% of total N) indicated that all LAB-treated rice straw silage except the control could be considered as near acceptable silage according to Wilkinson [23], who reported that optimally preserved silage must have an NH_3-N concentration <50 g/kg of total N. Haigh [24] stated that an NH_3-N value <100 g/kg N usually indicates successful preservation.

The new LAB strains in this study (1821 and 1841) decreased the acetic acid, and increased the lactic acid content of silages significantly ($p<0.05$). Some researchers have reported that use of a LAB inoculant increases lactic acid production and decreases silage pH, the acetic acid level, and butyric acid production [25,26]. The ratio of lactic acid to acetic acid is a good indicator of the type of fermentation undergone by the silage [27]. Zhang et al [27] also reported that a ratio of at least 2:1 indicates strong homolactic fermentation. The ratios of lactic acid to acetic acid in LAB 1821 and 1841 were higher (9.66 and 9.58) than those of a commercial inoculant ($p<0.05$). According to this result, LAB 1821 and 1841 are classified as homofermentative LAB.

Many studies have shown positive effects of bacterial inoculants on the digestibility of silage when fed to beef cattle [28], and dairy cattle [29]. Aksu et al [30] also reported that the digestibility of DM and NDF in inoculated corn silages were higher than those of a control. Other researchers have reported that inoculating LAB does not have positive effects on digestibility [26]. Although the average IVDMD of all rice straw silages was low in this study, all LAB significantly increased the IVDMD of silage ($p<0.05$).

CONCLUSION

Rice straw has low WSC and epiphytic LAB contents. A high-performance LAB must be inoculated to produce high-quality silage, and must grow well in a low sugar content environment. In this study, LAB 1821 met these requirements considering all criteria we assessed

CONFLICT OF INTEREST

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

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