Lactobacillus plantarum Improves the Nutritional Quality of Italian Ryegrass with Alfalfa Mediated Silage

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ABSTRACT

The present study was planned to analyze the nutritional quality, microbial counts and fermentative acids in Italian ryegrass (IRG) 80% and alfalfa 20% (IRG-HV) mediated silage inoculated with lactic acid bacteria (LAB) as a probiotic strain for 3 months. Crude protein (CP), acid detergent fiber (ADF), and neutral detergent fiber (NDF), total digestible nutrient (TDN) and *In-vitro* dry matter digestibility (IVDMD), lactic acid bacteria (LAB), yeast and fungi counts and fermentation metabolites such as lactic acid, acetic acid and butyric acids were analyzed. The result shows that the nutritional quality and metabolite profiles of silage were significantly improved with LAB. For microbial counts, LAB showed dominant followed by yeast as compared with control silage. The pH of the silage also reduced significantly when silage inoculated with LAB. The result confirmed that silage preparation using different crops with *L. plantarum* inoculation is most beneficial for the farmers.

(Key words : *Italian ryegrass*, Lactic acid bacteria, Nutritional quality, Microbes, Fermentative metabolites)

I . INTRODUCTION

Italian ryegrass (IRG) is an important material in the silage preparation because of IRG providing high yields and the ability to promote high forage intakes (Cooke et al., 2009).

IRG is a fastest growing grass in South Korea climate. However, alfalfa is not cultivated as a forage crop in Korea, although it is grown in nearly all environmental soil conditions. It has high yield and more quality. alfalfa contains 17~19% of crude protein content when 10% of plant is showing first bloom. Alfalfa is not as difficult to ensile as compared with other legumes, due to its greater dry matter content.

Silages play very important roles as preserved forages in most of the countries including South Korea, Japan and china. Ensiling is an important preservation method used for forage crops. The silage quality is based on production of organic acids which prevent the undesirable microbial growth under the anaerobic condition. Organic acid produced

by homo-and hetrofermentative lactic acid bacteria (LAB) through conversion of water soluble carbohydrate into organic acids especially lactic acid which is the main reason for reducing the pH of silage which inhibits the spoilage microbial growth and plant enzymes that could maintain the nutritive values of the silage (McDonald et al., 1991). Homolactic fermentation by *Lactobacillus* is more desirable than other types of fermentation because of the theoretical recoveries of dry matter and energy are higher.

The genus *Lactobacillus* is Gram-positive and mainly involved in the fermentation of dairy food products, food fermentation and food preservation (Giraffa et al., 2010). In general, natural populations of LAB in the plants are very low, which is not sufficient for improving the silage quality. Therefore, we need to add the additional lactic acid bacteria (LAB) during the silage preparation. These additions of LAB could enhance the production of fermentative acids and the quality of silage. Most commonly used homofermentative *Lactobacillus* in silage preparation are *Lactobacillus plantarum*, *L. rhamnosus*, *L. acidophilus*, *L. brevis*, *L.*

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fermentum, *L. reuteri*, *Pediococcus acidi lactici*, and *Enterococcus faecium* (Avila et al., 2010). Therefore, this study was carried out to investigate the quality and nutritive values in silage prepared by the combination of IRG and alfalfa using lactic acid bacteria as a probiotic strain by ensiling method.

II. MATERIALS AND METHODS

1. Collection and preparation of silage

IRG and alfalfa were harvested at flowering stage. Individually, one hundred grams of IRG, one hundred grams of alfalfa and eighty grams of IRG with twenty grams of alfalfa were packed in an air-diffusible bag. Silage was manufactured with the addition of fermentative additive containing *L. plantarum* procured from Chung-Mi Bio Co., Korea (CMBC). *L. plantarum* was dissolved by the method of CMBC and then sealed to prevent air flow. Each of the samples with or without strains was prepared in triplicate. Silage were stored in underground and opened after 3 months. The nutritive values, microbial counts and fermentative metabolites were analyzed.

2. Nutrient composition and analysis

Acid detergent fiber (ADF) and neutral detergent fiber (NDF) were analyzed according to (Van Soest et al., 1993). The content of crude protein was quantified by standard procedure (AOAC, 1990). The *in-vitro* dry matter digestibility (IVDMD) was analyzed by the modified method of Moore (1970). Total digestible nutrient (TDN) was calculated as follow; $88.9 - (ADF\% \times 0.79)$. Samples were powdered and passed through a 1 mm sieve prior to analysis.

3. Enumeration of microbial populations

Ten grams of wet silage samples were transferred into sterile flasks containing 100 mL of sterile water. Then it was kept in an orbital incubator shaker at 150 rpm for 30 min and then made a tenfold serial dilutions with water (Miller and Wolin, 1974). 0.1 mL of the sample was spread on selective media (Rogosa, and Sharpe agar (Diffco) and

Bromocresol purple blue agar medium) for enumeration of LAB colony incubation under the micro aerobic condition at $28 \pm 1^\circ\text{C}$ for 2 days. Yeasts and molds were enumerated on 3 M petrifilm (3 M Microbiology Products, St.Paul, USA), and following aerobic incubation at $28 \pm 1^\circ\text{C}$ for 2 days. Fungi were enumerated on Potato Dextrose agar (PDA) [4 g/L of potato starch (Diffco), 20 g/L of starch (Diffco), and 20 g/L of agar (Diffco)] following aerobic incubation at $28 \pm 1^\circ\text{C}$ for 3 days.

4. Analyses of fermentation metabolites

Weighing 20 g of silage and 80 ml of deionized water into a blender and homogenizing for 2×30 s. The homogenate was kept in a refrigerator at 4°C until centrifugation (8000 rpm at 4°C for 20 min). The pH of the supernatant was measured after centrifugation using a combination electrode. Water extracts were stored at -20°C with and without stabilization with 5% meta-phosphoric acid (final concentration). Fermentation byproduct lactic acid content was analyzed by HPLC (HP1100 Agilent Co. USA). The levels of acetic acid and butyric acid were analyzed by Gas chromatography (GC-450, Varian Co., USA) (Kristensen et al., 2007).

5. Statistical analysis

The results were statistically analyzed by one way ANOVA at $P=0.05$ with the help of SPSS 11.5 version software package.

III. RESULTS AND DISCUSSION

In the present study, we investigated the nutritive values, fermentative acids and microbial counts in the silage prepared by combining of IRG with alfalfa. In general, IRG and alfalfa were used as a feeds for the ruminant in South Korea and other countries. In Korea, IRG, rye, alfalfa and crimson clover were used for silage preparation. Farmers are very much interested in the preparation of silage using IRG, rye, barley with legumes, because less cost for preparation as compared to concentrated feed. However, their nutritive values were altered depending upon the preparation techniques. Therefore, livestock farmers prepared

silage by the addition of *L. plantarum* as a probiotic strain. After preparation, nutritive values and fermentation metabolites of silages were analyzed. It is confirmed that, silage preparations by ensiling method enhance the nutritional values, as evidenced by changes in the nutrient composition and increased the fermentative metabolites.

In present study, we analyzed the nutritive profiles of CP, ADF, NDF, TDN and IVDMD in control and experimental silage. The CP levels slightly increased when IRG silage prepared with lactic acid bacteria (LAB) (11.84%) as compared with control (10.17%), but CP content was observed in the alfalfa (19.48%) and alfalfa with LAB (19.74%) mediated silage. Silage prepared by a combination of 80% IRG and 20% alfalfa shows 12% CP content, but no significant changes were observed when the 80% IRG and 20% silage inoculated with LAB as compared with 80% IRG and 20% alone mediated silage. The content of CP significantly increases by combination of IRG with

alfalfa as compared to IRG alone, ADF and NDF levels were slightly changed in all experimental silage. There is no significant change of TDN and IVDMD were observed in all experimental silages as compared with respective control (Table 1).

The number of bacteria, yeast and fungi colonies in the experimental silages was presented in the Table 2. IRG alone mediated silage shows 104×10^7 CFU and IRG with LAB inoculated silages exhibited the 159×10^7 CFU. The addition of LAB to the alfalfa during silage preparation increased the LAB population as compared with alfalfa alone. Inoculation of LAB to IRG and alfalfa mediated silage showed more number of LAB colonies as compared with control. For yeast counts show slight variation in the experimental silages, but no fungi were observed in the both control and experimental silages. The total number of LAB, yeast and fungi were coinciding with previous reports on the epiphytic micro flora of corn (Lin et al., 1992).

Table 1. Changes of nutritive value and *in vitro* dry matter digestibility on mixture silage of IRG- alfalfa according to inoculation of lactic acid bacteria

Treatment	CP ³⁾ (%)	ADF ⁴⁾ (%)	NDF ⁵⁾ (%)	TDN ⁶⁾ (%)	IVDMD ⁷⁾ (%)
IRG ¹⁾	10.17 ^b	37.68 ^a	59.14 ^a	59.13 ^b	67.20 ^b
IRG + LAB ²⁾	11.84 ^b	36.02 ^a	53.60 ^a	60.44 ^b	67.72 ^b
Alfalfa	19.48 ^a	32.78 ^b	41.01 ^b	63.00 ^a	73.93 ^a
Alfalfa + LAB	19.74 ^a	33.89 ^b	42.01 ^b	62.13 ^a	71.78 ^a
Alfalfa + IRG	12.00 ^b	35.06 ^a	53.40 ^a	61.20 ^b	71.54 ^{ab}
Alfalfa + IRG + LAB	12.00 ^b	36.29 ^a	55.20 ^a	60.23 ^b	70.97 ^{ab}

¹⁾ IRG: *Italian ryegrass*, ²⁾ LAB: Lactic acid bacteria, ³⁾ CP: Crude protein, ⁴⁾ ADF: Acid detergent fiber, ⁵⁾ NDF: Neutral detergent fiber, ⁶⁾ TDN: Total digestible nutrient, ⁷⁾ IVDMD: *in vitro* dry matter digestibility.

^a and ^b: Means with different letters within a column are significantly different at the 5% level.

Table 2. Changes of microbes on mixture silage of IRG-alfalfa according to inoculation of lactic acid bacteria

Treatment	LAB ²⁾ ($\times 10^7$ CFU/g)	Yeast ($\times 10^4$ CFU/g)	Fungi ($\times 10^4$ CFU/g)
IRG ¹⁾	104.00 ^b	14.50	0.00
IRG + LAB ²⁾	159.00 ^b	10.20	0.00
Alfalfa	167.00 ^b	21.50	0.00
Alfalfa + LAB	284.00 ^a	21.50	0.00
Alfalfa + IRG	156.00 ^b	15.00	0.00
Alfalfa + IRG + LAB	284.00 ^a	21.50	0.00

¹⁾ IRG: *Italian ryegrass*, ²⁾ LAB: Lactic acid bacteria, ³⁾ CFU: Colony per unit.

^a and ^b: Means with different letters within a column are significantly different at the 5% level.

Table 3. Changes of pH and organic acids on mixture silage of IRG-alfalfa according to inoculation of lactic acid bacteria

Treatment	pH	Lactate (%/DM ³⁾)	Acetate (%/DM)	butyrate (%/DM)	Flieg's score
IRG ¹⁾	4.09 ^a	7.39 ^b	1.10 ^b	0.74	Excellent
IRG + LAB ²⁾	3.70 ^a	10.21 ^a	0.15 ^c	0.04	Excellent
Alfalfa	5.03 ^b	4.52 ^c	4.52 ^a	0.09	Excellent
Alfalfa + LAB	5.03 ^b	5.14 ^c	3.83 ^a	0.05	Excellent
Alfalfa + IRG	4.11 ^a	6.03 ^b	2.05 ^b	0.08	Excellent
Alfalfa + IRG + LAB	4.32 ^a	7.28 ^b	1.58 ^b	0.44	Excellent

¹⁾ IRG: *Italian ryegrass* ²⁾ LAB: Lactic acid bacteria ³⁾ DM: Dry matter.

^{a,b} and ^c: Means with different letters within a column are significantly different at the 5% level.

Silages had substantially greater counts of LAB, yeast, and fungi than plant samples. (McDonald et al., 1991). Corn and sorghum silages are susceptible to aerobic deterioration, especially in warm climates (Filya, 2003). This is due to aerobic yeasts which are most active at 25~30°C (Ashbell et al., 2002). Therefore, need to add a natural supplement for inhibition of undesirable microorganism and improving the nutritive values. Lactic acid bacteria enhance the aerobic deterioration of whole cereal silage and inhibit the fungi growth (Weinberg et al., 1996). Acetic acid plays an important role in the aerobic stability of silage (Danner et al., 2003). The simultaneous production of lactic acid and acetic acid together with low residual butyric acid is important factors to reach aerobic stability in silages (Danner et al., 2003).

Here, we added LAB for silage preparation, which reduce the pH of silage and enhances the fermentative products as compared with control. Addition of LAB to the IRG and alfalfa mediated silage showed reduced pH level and increased the lactic acid production followed by acetic acid and butyric acid as compared with respective control (Table 3). The use of *L. plantarum* has been shown to further improve the fermentation and quality of silage, resulting in increased the lactic acid production (Ojeda and Montejó, 2001).

IV. CONCLUSION

Inoculation of lactic acid bacteria to IRG and alfalfa mediated silages were improved the nutritive values, reduced the pH of the silage and enhance the production of

fermentation products, which is a very important advantage for the silage preparation because of fermentative products will be declined the pH of the silage which inhibits the undesirable microbial growth and improved the quality of the silage for long time storage purpose. Therefore, isolation of high cell density LAB would be useful for the preparation of high quality silage and will be providing new economic technology for effective implementation for solving the ruminant feed demand in the growing countries.

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