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Research Article

Dual Culture Inoculation Enhanced Quality of Silage Produced from Leguminous Plants

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ABSTRACT

Ensiling is the most preferred technology to preserve the silage quality with high nutrients by the presence of lactic acid bacteria. In this study, lactic acid bacteria RJ1 and S22 were used to make the silages from different leguminous plants such as alfalfa, hairy vetch and red clover. Experimental groups were divided into control and LAB inoculated groups. LAB inoculated group; all legumes treated with a mixture of RJ1 and S22 and made an anaerobic condition for 45d. Without the addition of LAB considered the control group. The results showed that the lactic acid content was higher in all silages in response to LAB treatment and acetic acid content was slightly increased except red clover by LAB compared to control silages. A poor silage quality marker butyric acid was reduced all legume silages in response to LAB inoculation than control silages. The organic acid is closely associated with microbial population experimental silages. We noted that higher LAB and lower yeast were found in the silage in response to LAB treatment. The contents of crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF), and total digestible nutrient (TDN) were not altered significantly between control and LAB treated silages. Overall data suggested that the inclusion of additional LAB potentially enhance the silage quality and preserved the nutrients for long period.

(Key words: Ensiling, Legumes, Lactic acid bacteria, Silage, Preservation)

Ⅰ. INTRODUCTION

The lactic acid bacteria play a major role in the production of high-quality silages via controlled fermentation(Ávila and Carvalho, 2020). The LAB could utilize the water-soluble carbohydrates and converts them into valuable organic acids. It's well known that, the lactic acid is major components in the fermented silages among the organics acids. Also, LAB can produce a significant amount of acetic acid and either inhibit or reduce the butyric acid content of silages. A higher butyric acid level indicates poor silage quality. Production of essential organic acids by LAB could contribute to reduce pH that helps to control/inhibit the growth of undesirable microbes such as yeast, mold and bacterial species *Clostridium* and *Enterobacter*. A higher population of these undesirable microbes can compete for nutrient utilization with LAB, resulting in higher pH and butyric acid content, enhanced proteolytic activity, lower lactic acid, resulting in poor quality silages(Silva et al., 2016; Soundharrajan et al., 2020). The use of LAB prevents the reduction of dry matter loss (DM), increases in the production of microbial metabolites, and improvement of microbiological and nutritional quality (Muck et al., 2018; Oliveira et al., 2013; Wilkinson and Rinne, 2018). Therefore, extensive studies and interests have been increased in the use of LAB as additives in silages production worldwide. In general, LABs are present in plants and other substrates and are used for silage fermentation naturally. However, they are often present in very low numbers (Cai, 1999; Carvalho et al., 2021; Fabiszewska et al., 2019). It is not sufficient to induce the vigorous fermentation process which leads to increase pH of silage and lower the essential acids production hence; the addition of LAB is required when ensiling process.

The utilization of leguminous forages to livestock has been considered the most prominent nutrients to improve animal production, the efficiency of nutrient utilization and reduce the dependency on imported and higher carbon footprint feeds. Legume forages could be fed with animals by direct grazing, or housing by conserved hay or silages. The potential of legumes for direct

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grazing is limited due to their susceptibility to crushing and the preferential grazing by livestock (Phelan et al., 2015). In addition, fresh feeding of legumes is also limited by the rain season and reduced the protein-rich feedstuffs during the dry season. Another issue in legumes feeding as hay may be limited by the loss of dry matter occurring while drying, transport and forage (Castro-Montoya and Dickhoefer, 2018). So, due to the above said limitations ensiling method with LAB is considered as the most essential tool to conserve the leguminous based animals feed with high nutrients for a long time. In this study, we prepared silage from different legumes such as alfalfa, hairy vetch and red clover using LAB as an additive. In general, legume has higher crude protein with buffer capacity and lower water-soluble carbohydrate (WSC). It is hard to develop high-quality silages because WSC is essential for LAB growth which could be used it and converted into essential organic acid. The selected strains had tolerant to growth in low carbohydrate sources(Kuppusamy et al., 2020); hence we used these strains for legume silage production.

Ⅱ. MATERIAL AND METHODS

1. Cultivation of legumes and place

Alfalfa (*Medicago sativa*), Hairy vetch (Hung villosa) and Red clover (Red Quin) were grown at grassland and forage cultivation field, National Institute of Animal Science, Seonghwan-eup, Cheonan, South Korea All legumes were harvested at the mid- flowering stage. All legumes were cultivated by standard grassland cultivation guidelines given by Rural Development Administration recommendations.

2. Lactic acid bacteria (LAB)

The LAB (RJ1 and S22) were isolated from rumen fluid of Hanwoo steer cattle collected from livestock animal farm, National Institute of Animal Science, Seonghwan-eup, Cheonan, South Korea. *Lactobacilli* MRS agar was used to isolate LAB by a tenfold serial dilution method and bromocresol purple (BCP) agar was used to confirm its identity of LAB. The isolated strains were characterized and identified by biochemical and molecular methods (Kuppusamy et al., 2020).

3. Bacterial cultivation and Enumeration

The LABs were inoculated in MRS broth (CONDA, Madrid, Spain) and incubated at 30±2 ○C for 24 h. Aliquot volume (1mL) of culture broth was centrifuged at 4000x for 10 minutes and the supernatant was discarded. The pellets were washed twice with phosphate-buffered saline (PBS, pH 7.4). The bacterial colonies were enumerated by the QUANTOMTM cell staining method (Logos biosystem, Gyeonggi-do, Korea) (Soundharrajan et al., 2020). Simultaneously, the remaining portion was centrifuged at 4000x for 10 minutes. Then collected pellets were washed with PBS twice and diluted in water for silage production.

4. Silage production from leguminous plants

The legumes such as the alfalfa (middle flowering), hairy vetch (middle flowering) and red clover (middle flowering) were harvested in Grassland and Forage Farm, National Institute of Animal Science, Cheonan, South Korea. The harvested legumes were dried under field conditions and then the moisture content of samples was analyzed frequently. After reaching the expected moisture content, then weighed 200g for each legume and chopped to a theoretical cut of 1.5-2.5 cm with a hand operated chaff cutter. The samples (250gram each) were packed in a silage bag (28×36 cm, Aostar Co., Ltd., Seoul, Korea). The samples were divided into two groups (each two replicates); control and LAB treatment. The treatment group was inoculated with LAB mixture (*Lactobacillus plantarum*-RJ1 and *Pediococcus pentosaceus*-S22) at the density of 10^5CFU/ bag (5×10^4CFU/ bag from each inoculant). Without inoculants is considered as a control group. The air was evacuated from all bags by a vacuum sealer (Food saver V48802, MK Corporation, Seoul, Korea). All vacuum sealed bags were kept at room temperature for 45 days. After opening at day 45, pH and nutrient profiles such as CP(AOAC, 1990), ADF (AOAC, 2000), NDF (Van Soest et al., 1991), and TDN (TDN = 89.9 − (ADF * 0.79)(AOAC, 1990) contents of silage were determined.

5. Quantification of organic acids and microbial population enumeration in ensiled silages

Ten grams of silage samples from the experimental group of legumes were taken and mixed with 90 mL sterile water and shake vigorously in an orbital shaker for 60 minutes. The extract

was filtered via double layers of sterilized cheesecloth and divided into three portions. A portion was used to analyze the pH of silage samples (lab pH meter, Thomas Scientific, Swedesboro, NJ, USA). Other portions were used to determine the content of organic acids by the HPLC method (C18 column, Agilent HPLC 1100 mobile phase 0.1mM phosphoric acid; Flow rate 0.5mL/min; detector wavelength 220nm; injection volume 10μL (Arasu et al., 2014) and LAB was enumerated by MRS agar after 48hrs, yeast and mold were counted after 72hrs by 3M petriflim (3M Microbiology Products, USA)(Soundharrajan et al., 2020).

6. Statistical analysis

Data were analyzed using t-test using SPSS software (SPSS ver.16.0, SPSS Inc., IBM Corp., Armonk, NY, USA). The means were considered statistically significant at $P \leq 0.05$.

Ⅲ. RESULTS

1. LAB on alfalfa

Table 1 shows the moisture, pH and organic acid contents of experimental silages. The moisture contents of silage were 58.02% and 57.79% for control and LAB treated silages, respectively. The addition of LAB reduced the pH of silage (pH 4.62) than the control silage (pH 5.13). The level of lactic acid content for control and LAB treated silages was 0.56% and 2.47% respectively. The acetic acid content was slightly increased in silages treated with LAB than the control silage (control % 0.29 vs LAB 0.52). The butyric acid content for LAB treated and control silages were 0.01% and 0.05%, respectively. The content of crude protein (CA) (20.34% vs 21.71%), ADF (30.06% vs 26.22%), NDF (37.05% vs 33.74%) and TDN (64.72% vs 68.19) in control and LAB treated silages (Table 2). The microbial population in experimental silages had lower LAB (6.8 log10 CFUg-^{1}), and higher yeast counts (3.9 log10 CFUg-^{1}) in non-inoculated silages whereas silage treated with LAB had higher LAB with lower yeast counts. We did not detect any mold growth in both control and LAB treated silages.

2. LAB on hairy vetch

The moisture contents of hairy vetch silages were 51.19 % for control and 47.94% for LAB treated silage. The addition of LAB to hairy vetch during ensiling process reduced pH compared to control silage. A higher lactic acid level was found in the silages treated with LAB compared to the control. Acetic acid level slightly increased and decreased butyric acid content of silages in response to LAB treatment compared to control silages (Table 3). The CP, ADF, NDF and TDN content of silages remain the same in both experimental groups. Increased LAB and decreased yeast counts were noted in silages treated with LAB mixture than the control silage. We did not have any mold growth in all the experimental silages (Table 4).

3. LAB on red clover

The moisture content of red clover silages was 64.81% for control and 67.14% for LAB inoculated silages. The addition

Table 1. Organic acid profiles of experimental silages of alfalfa

Groups	Moisture (%)	pH	LA (%/DM)	AA (%/DM)	BA (%/DM)
Control	58.02 ± 0.74	5.13 ± 0.15^{a}	0.56 ± 0.06^{b}	0.29 ± 0.02	0.05 ± 0.01
LAB	57.79 ± 0.45	4.62 ± 0.03^{b}	2.47 ± 0.05^{a}	0.52 ± 0.04	0.01 ± 0.00

Control group: legume silage produced without inoculant; LAB group: Legume silage produced with LAB (*Lactobacillus plantarum*-RJ1 and *Pediococcus pentosaceus*-S22). LAB: Lactic acid bacteria; LA: Lactate; AA: Acetate; BA: Butyrate.

Table 2. Nutrient profile of experimental silages of alfalfa

Groups	CP (%DM)	ADF (%DM)	NDF (%DM)	TDN (%DM)	LAB	Yeast
Control	20.34 ± 0.64	30.06 ± 1.30^{a}	37.05 ± 0.65	64.72 ± 1.09	6.8 ± 0.04^{b}	3.9 ± 0.05^{a}
LAB	21.71 ± 0.48	26.22 ± 0.78^{b}	33.74 ± 1.49	68.19 ± 0.62	8.2 ± 0.03^{a}	3.3 ± 0.07^{b}

DM: dry matter content; CP: Crude protein; ADF: Acid detergent fiber: NDF: Neutral detergent; TDN: Total digestible nutrients; LAB (log10 CFUg-^{1}); yeast (log10 CFUg-^{1}). The data represent the mean ± SEM (p value 0.05 level) ^{ab}p<0.05 alphabets within columns indicate significant differences between experimental silages.

Table 3. Organic acid contents of experimental hairy vetch silages

Groups	Moisture (%)	pH	LA (%/DM)	AA (%/DM)	BA (%/DM)
Control	51.19 ± 0.10	4.99 ± 0.07[a]	0.48 ± 0.02[b]	0.25 ± 0.01	0.04 ± 0.01
LAB	47.94 ± 4.46	4.14 ± 0.06[b]	1.83 ± 0.03[a]	0.38 ± 0.01	0.01 ± 0.00

Control group: legume silage produced without inoculant; LAB group: Legume silage produced with LAB (*Lactobacillus plantarum*-RJ1 and *Pediococcus pentosaceus*-S22). LAB: Lactic acid bacteria (RJ1 and S22); LA: Lactate; AA: Acetate; BA: Butyrate.

Table 4. Nutrient profile of experimental hairy vetch silages

Groups	CP (DM %)	ADF (DM %)	NDF (DM %)	TDN (DM %)	LAB	Yeast
Control	23.1 ± 0.67	31.26 ± 0.73	46.30 ± 0.80	64.20 ± 0.63	7.73 ± 0.13[b]	2.1 ± 0.56
LAB	23.4 ± 0.02	31.10 ± 0.80	43.80 ± 0.88	64.33 ± 0.07	8.11 ± 0.19[a]	N/A

DM: dry matter content; CP: Crude protein; ADF: Acid detergent fiber: NDF: Neutral detergent; TDN: Total digestible nutrients; LAB (log10 CFUg-^{1}); yeast (log10 CFUg-^{1}). The data represent the mean ± SEM (p value 0.05 level) [ab]$p<0.05$ alphabets within columns indicate significant differences between experimental silages.

Table 5. Organic acid changes in red clover silages in response to LAB treatment

Groups	Moisture (%)	pH	LA (%/DM)	AA (%/DM)	BA (%/DM)
Control	64.81 ± 0.58	4.99 ± 0.61[a]	3.22 ± 0.08[b]	0.72 ± 0.01	0.20 ± 0.00
LAB	67.14 ± 1.39	3.92 ± 0.04[b]	5.41 ± 0.05[a]	0.55 ± 0.04	0.06 ± 0.04

Control group: legume silage produced without inoculant; LAB group: Legume silage produced with LAB mixture (*Lactobacillus plantarum*-RJ1 and *Pediococcus pentosaceus*-S22). LAB: Lactic acid bacteria (RJ1 and S22); DM: dry matter content; LA: Lactate; AA: Acetate; BA: Butyrate.

Table 6. Changes in nutrient profile of control and LAB inoculated red clover silages

Groups	CP (DM %)	ADF (DM %)	NDF (DM %)	TDN (DM %)	LAB	Yeast
Control	21.14 ± 0.77	21.93 ± 0.18	26.91 ± 1.38	71.57 ± 0.14	6.90 ± 0.02[b]	3.6 ± 0.06
LAB	21.19 ± 0.024	23.35 ± 0.17	31.71 ± 1.04	70.45 ± 0.14	7.90 ± 0.05[a]	2.9 ± 0.55

DM: dry matter content; CP: Crude protein; ADF: Acid detergent fiber: NDF: Neutral detergent; TDN: Total digestible nutrients; LAB (log10 CFUg-^{1}); yeast (log10 CFUg-^{1}). The data represent the mean ± SEM (p value 0.05 level) [ab]$p<0.05$ alphabets within columns indicate significant differences between experimental silages.

of LAB did not alter the pH of the silages compared to control silages whereas higher lactic acid content was found in the silage treated with LAB. LAB reduced the acetic acid and butyric acid content of silage than the control silage (Table 5). The CP, ADF, NDF and TDN contents of silages remain the same in both experimental groups. Microbial population enumeration results showed higher LAB and lower yeast counts in silage treated with LAB inoculated silage than the control (Table 6).

Ⅳ. DISCUSSION

The quality of silage is closely associated with successful fermentation by lactic acid bacteria which preserved the nutrition for a long time. Ensiling is a process to preserve plant-based materials to provide year-round feed for livestock (Chen et al., 2021). It could help to increase silage quality by improving odor and taste (Yang et al., 2021). Preservation of forages for a long time is challengeable due to nutrients loss by oxidation, enterobacteria growths, proteolytic activity, undesirable fermentation, deamination and decarboxylation of amino acids because it also affects the forage and silage quality by increasing energy and accumulation of anti-nutritional compounds in forage (Borreani et al., 2018; Oliveira et al., 2017). In the present study, silages were produced from legume plants such as alfalfa, hairy vetch and red clover with biological additive lactic acid bacteria RJ1 and S22 mixture by an ensiling method. The reduction in pH indicates the successful fermentation of silages. An addition of LAB mixture to legumes forage during ensiling showed a significant reduction in pH of all legumes silage compared to control silage, but ranges of pH

had varied among silages. The maximum reduction of pH was noted for the red clover silages followed by hairy vetch and alfalfa. The final pH values of all silages were within the range of 4.48 -4.88 which is considered sufficient for legumes silage (Heinritz et al., 2012). Similar results were noted for all legumes silages treated with LAB mixture. The addition of LAB is intended to ensure the fermentation process via increasing organic acid content of silages particularly lactic acid was dominant acid in the fermented alfalfa silage than the other organic acids that reduced the pH of silages(Nascimento Agarussi et al., 2019). Another report claimed that rapid acidification and higher lactic acid production was achieved in legume silage in response to fresh inoculant compared to freeze-dried inoculant treatment (Kizilsimsek et al., 2007). In the present study, we used fresh bacterial inoculants (24hr culture) for alfalfa silage production. It showed that a higher amount of lactic acid (>4 fold vs control) in silage compared to other legume silages. Lactic acid content at fold wise (1.6 fold vs control) was found lower in red clover silage than the legume silages. Even though red clover silages had lower pH than the others, the fold of lactic acid content was lowered due to control silage had a significant level of lactic acid. A moderate level of lactic acid (3.8 fold vs control) was found in hairy vetch treated with LAB mixtures compared to alfalfa and red clover silage. The acetic acid content was slightly increased in alfalfa and hairy vetch silages than the control silages whereas red clover silage had lower acetic acid content than the control silage. A slight increase in the acetic acid level of silages is generally acceptable in silage production. Acetic acid is a sole substance responsible for the improvement of silage stability and it acts as an inhibitor of spoilage microorganisms(Danner et al., 2003). Butyric acid is a key negative marker in silage production. A higher level of butyric acid in ensiled silage indicates poor quality and aerobic stability loss. In our study, the addition of the LAB mixture significantly reduced the production of butyric acid in all legumes compared to control silages. Similar responses (>4 fold vs control) by LAB on butyric acid content were noted in the alfalfa and hairy vetch silages. But in red clover, we noted a lower reduction in butyric acid level (>3 fold vs control) in silages treated with LAB mixture compared to other legumes. It indicates added inoculants had a significant ability to improve the legume silage quality via the production of essential organic acids and lowered pH. The organic acids profiles of silages significantly correlated with the microbial population in experimental silages. The legume silages treated with mixed LAB showed a higher LAB population than the control. It indicates that the RJ1 and S22 could compete with other undesirable microbial growths as evidenced by a reduction in yeast population in LAB treated silages than the control silages. It confirmed that RJ1 and S22 could play a major role in the fermentation of alfalfa, hairy vetch and red clover silages. Similarly, different types of LAB inhibit growths of mold and yeast and increased lactic acid content of legumes silage (Zielinska et al., 2015). In addition, CP, ADF. NDF and TDN level was not altered significantly in silages treated with LAB mixture compared to control silages. Added LAB even though not provided a positive impact on nutritive profiles of silages but did not alter the native form of all silages. It confirmed the efficiency of inoculants to improve the silage quality by positive fermentation without altering its native form. Several researchers have confirmed the addition of LAB inoculants significantly improved and preserved the legumes silage quality via rapid acidification and higher production of lactic acid and inhibition of undesirable microbial growths (Guo et al., 2020; Jiang et al., 2020; Kaldmäe, 2009; Schmidt et al., 2009; Silva et al., 2016)

Ⅴ. CONCLUSION

The *Lactobacillus plantarum* RJ1 and *Pedioccocus pentosaceus* S22 were used as a mixed additive to develop silages from different leguminous plants. The addition of mixed LAB strongly reduced the pH of all legume silages. Stronger productions of lactic acid with marginal amounts of acetic acid were noted silages in response to inoculant treatment. Butyric acid content was significantly reduced compared to control silages. The pH and organic profiles are closely associated with the microbial population of silages. The higher LAB and lower yeast were found in the inoculated silages. It suggested that both RJ1 and S22 have the potential ability to induce lactic acid fermentation, could be used as the best additives for various legume plants.

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