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Research Article

Silages of Rye Harvested at Different Stages: A Study on Microbial Inoculants Responses in Improving Rye Silage Fermentation Quality

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ABSTRACT

The present study analyzes the role of Lactic Acid Bacteria Mixture (LBM) on improving rye silage quality. Rye of four different stages (Booting, Heading, Flowering, and Late flowering) was collected and silage was prepared. The nutrient profile analysis of experimental silage groups showed no significant changes between control and LBM inoculation. Interestingly, the pH of rye silage in LBM treatments showed significant reduction than control (p<0.05) in all stages of rye silage. However, lowest pH (3.69) resulted on booting stage among other stages of rye. Subsequently significant lactic acid production was noted in all stages of LBM inoculation than control. Conversely maximum lactic acid production of (5.33%DM) was noted at booting stage followed by (4.86%DM) in heading stage. Further the lactic acid bacterial (LAB) count in LBM inoculated group showed significant increase than control. Similarly, the silage of booting stage group registered maximum LAB population (63.7 $\times 10^6$CFU/g) after that heading stage (32.3 $\times 10^6$CFU/g). Further significant reduction in yeast growth and no fungal growth was noted in all LPM treatment groups. Hence, LBM inoculants could be a better additive for improving rye silage quality.

(**Key words** : Rye, Heading stage, Booting stage, *L.plantarum*, Lactic acid)

Ⅰ. INTRODUCTION

Rye is a potential stress tolerant crop. It can withstand undesirable growing conditions like deep winter, low fertility soils, sandy soils, and extreme drought climate. Rye requires the smaller amount of water (30%) than other forage crops. The rye production offers greater economic returns for small land holding farmers as well as large landholders. Winter rye shows better agronomic characteristics than spring verities. Further rye is the one of the primary cattle feed in Korea. Subsequently, food production is a primary need for the global population and that is the reason for the significant part of soil has been allocated to agricultural practices worldwide. Among these agricultural practices, some portion has been allocated for animal feed production as grassland (Conant et al., 2001). Researchers predicted that during 2000-2050 the world population will increase to nine billion. Hence pastures play significant role in agriculture for the increase of meat and milk production to provide food for the raising human population (Kingston-Smith et al., 2012; Young et al., 2013)(Kingston-Smith et al., 2012; Young et al., 2013). The usage of pastures as animal feed that decrease the purchase of concentrated feed and simultaneously increasing the profitability for farmers. Further, knowledge in forage preservation can allow development in the quality of forage crops that turns to enhancement ruminant performance(Jacobs, 2014; Stott and Gourley, 2016).

Preservation of dairy products using fermentation is a classic method that dated 1000 years old technique(Rhee et al., 2011). Similarly, preserving forage by fermentation is of a primary need to compensate feed shortage caused by low pasture growth. Forage crops are known to contain water soluble carbohydrates that facilitate the homofermentative process and production of organic acids during ensilation process. The ensilation process comprises the combination of both aerobic and anaerobic environment with fermentation of carbohydrates by microbial inoculants (Muck, 2010). Microbial

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inoculants require various nutritional factors for growth at silo conditions. However they grow rapidly, improve the fermentation quality of forages in the silo conditions without influencing in ruminant performance and become dominant microorganisms on the crop.

Addition of microbial inoculants like lactic acid bacteria to the silo reduces the pH of the silage through lactic acid production that inhibits the growth of yeast and mold species (Ni et al., 2015). Based on organic acid production the lactic acid bacteria have been grouped as homofermenter and heterofermenter. In addition, lactic acid bacteria dominate the silage microbial profile using hydrogenperoxide production and anaerobic condition. However the anaerobic condition might be the main reason for the rapid lactic acid bacterial growth at insilo conditions (Muck, 2010). The objective of this study was to estimate the role of lactic acid bacteria mixture (LBM) on the fermentation quality and nutritive value of rye harvested at different stage.

II. MATERIALS AND METHODS

1. Preparation of Lactic acid bacteria mixture (LBM)

L. plantarum KCC-10, KCC-19 and K.46 were isolated and characterized by biochemical and molecular tools (Valan Arasu et al., 2013; Arasu et al., 2014; Valan Arasu et al., 2015). The LBM was prepared according to the manufacturer protocol, Top Silage private Limited, Korea. Accordingly, fresh culture of *L.plantarum* KCC-10, *L. plantarum* KCC-19 and *L. plantarum* K-46 were inoculated in mass cultivation medium containing glucose 1%, soy peptone 0.25%, yeast extract 1%, MgSO4 0.01%, $MnSO_4$ 0.04%, NaCl 0.1%, $CaCO_3$ 0.2%, Na_2HPO_4 0.6% in a fermenter. After mass cultivation, the cultures were freeze dried. This mixture of KCC-10, KCC-19, and K-46 (LBM) was used as inoculants in rye silage preparation.

2. Collection and preparation of silage

Rye was harvested at four different stages such as booting (10^{th} April), heading (21^{st} April), flowering (28^{th} April), and late flowering stage (11^{th} May), National Institute of Animal Science- RDA, Cheonan. The experiment was divided in to control group lactic acid bacteria mixture (LBM) inoculated group for each stage. Two hundred grams of rye was taken for each experimental group of different stages and was packed in different air diffusible bags. Subsequently, silage was prepared with the addition LBM inoculants. Each LBM inoculants ($1.5x10^{10}$cfu/g) was dissolved in distilled water (0.004g/10ml) and sprayed in to the 200g of rye in air diffusible bag. Silage was sealed to maintain anaerobic condition. Each of the samples with or without strains was prepared in triplicates. Silage was stored in room temperature for 45 days. The nutritive values, microbial counts and fermentative metabolites were analyzed.

3. Rye silage Nutrient profile analysis

Silage samples were ground well and passed through a 1 mm sieve prior to nutrient profile analysis. The crude protein (CP) was estimated by standard procedure (AOAC, 1990). Acid detergent fiber (ADF) and Neutral detergent fiber (NDF) were estimated according to the protocols described by (Soset et al., 1993). Total digestible nutrient (TDN) was calculated as follow; 88.9-(ADF% × 0.79) (Holland et al., 1990).

4. Microbial profile analysis

Ten grams of wet silage samples were transferred into sterile flasks containing 90 mL of sterile water. The sample was kept in an orbital shaker with incubator at the rotation speed of 150 rpm for 30 min. Subsequently, tenfold serial dilution was made using distilled water (Miller and Wolin, 1974). For the counting of LAB colony, the diluted sample (0.1 mL) was spread on selective media (Rogosa, and Sharpe agar (Diffco) and Bromocresol purple blue agar medium) and incubated at 28 ± 1℃ for two days. Yeasts and molds were counted on 3 M petrifilm (3 M Microbiology Products, St.Paul, USA), and followed by aerobic incubation at 28 ± 1℃ for four days. Fungi colonies were counted by using Potato Dextrose agar (PDA) [4 g/L of potato starch (Diffco), 20 g/L of starch (Difco), and 20 g/L of agar (Difco)] following aerobic incubation at 28 ± 1℃ for four days.

5. Estimation of organic acids

10 g of rye silage sample of four different stages was weighed, mixed with 90 ml of deionized water and kept in a refrigerator at 4℃ for 24 hr. After that, the samples were filtered through the filter paper (Whatman No. 6) and the filtrate was re-filtered using 0.22㎛ syringe filter before injection of samples in high-performance liquid chromatography (HPLC; HP1100,. Agilent Co. USA). The pH of the supernatant was measured after centrifugation using a combination electrode. The filtrate was stored at −70℃ with and without stabilization with 5% meta-phosphoric acid (final concentration). Lactic acid content was analyzed by HPLC. The contents of acetic acid and butyric acid were analyzed by Gas Chromatography (GC-450, Varian Co., USA) method described by Kristensen et al. (2007).

6. Statistical analysis

Samples were analyzed in three replicates and analysis of variance were performed on all the variables measured using SPSS/PC (Statistical Package for the Science, ver 12.0. USA). The T-test was used to determine the effect of inoculants on the quality of rye silage. Tests were run at the 5 % probability level.

III. RESULTS AND DISCUSSION

This study, deals with the role *L. plantarum* mixture (LBM) inoculants on improving the quality of rye silage harvested at different stages. The parameters such as organic acids, microbial counting and nutrient profile play main role in deciding silage quality. Accordingly, the nutrient profile examination of rye silage of different stages has been presented in (Table 1). The highest crude protein concentration (20.52%) was observed LBM mixture treated silage of booting stage. However no significant (P>0.05) changes were noted between the other stages of rye silage when compared to booting stage. According to (Cezário et al., 2015) the early stage forages rich in protein content but it reduces rapidly when it matures. Further there is a slower reduction in protein content of the leaf when compared to other parts of forage. On the other hand, the increased ADF and NDF were observed in late flowering stage silage (67.22% and 44.7%) and this result agreement with that the nutritional quality of forage decreases progressively with the increase in maturity of plant . The steep increase in the ADF, NDF and decrease in TDN level at late flowering stage further confirmed the highest nutrient availability at early stages of harvest than later stage of rye harvest (Bayble et al., 2007; Subhalakshmi et al., 2011).

The fermentative acid estimation results of rye silage have been presented in (Table 2). Subsequently, Low pH of 3.69 was observed at the booting stage of rye silage. Further, there was significant difference in pH changes was noted between the control and LBM inoculated group. Steep decrease in the pH of rye silage from late flower stage to booting stage (4.21-3.69) indicated the availability of higher dissolved nutrients in early stages of forage than the mature stage (Santos et al., 2011; Ferreira et al., 2014). Because of

Table 1. Effect of Harvesting date and inoculation of lactic acid bacteria on nutritive values of rye silage

Harvesting date	Inoculation	CP[2] (%)	ADF[3] (%)	NDF[4] (%)	TDN[5] (%)
Booting Stage	Control	20.72	46.33	26.99	67.58
	LBM[1]	20.52	48.34	27.92	66.84
Heading stage	Control	16.75	58.64	35.75	60.66
	LBM	16.2	58.16	34.95	61.29
Flowering stage	Control	15.39	58.88	37.95	58.92
	LBM	15.53	59.69	37.73	59.09
Late-flowering stage	Control	7.39	68.25	42.39	55.41
	LBM	7.53	67.22	44.7	53.59

[1]LBM: Lactic acid bacteria mixture [2]CP: Crude protein, [3]ADF: Acid detergent fiber, [4]NDF: Neutral detergent fiber, [5]TDN: Total digestible nutrient

availability of higher nutrients at early stage of forage there is possibility to the growth of Clostridium species using lactic acid produced by the fermentation of dissolved carbohydrate (Ribeiro et al., 2014; Arzate-Vázquez et al., 2016). Hence it is necessary to maintain the anaerobic condition that allows the rapid growth of Lactic acid bacterial strains and reduction in the silo pH (Borreani et al., 2008). This condition inhibits the growth of undesirable organisms and increases the aerobic stability of the silage(Tabacco et al., 2011). Similarly there is significant increase in the lactic acid content was noted in LBM inoculated groups than control at all stages of rye silage. The lactic acid bacteria like *L.buchneri* can to convert lactic acid in to acetic acid. This activity will help to maintain the pH level of the silage and thus provide aerobic stability (Filya et al., 2006). In this experiment the LBM inoculated group showed moderate increase in the acetic acid level but no significant changes observed when compared to control group. Hence this confirmed the acetic acid producing nature of LBM inoculants. Further the reduction of final pH of silage by homofermentative inoculants through acetic acid production will inhibit the production of butyric acid (Danner et al., 2003). Accordingly no butyric acid production was noted in LBM inoculated group.

The enumeration of microbial population has been listed in (Table 3). The fermentation of sugars by lactic acid bacteria is the main criteria of the ensiling process(Ni et al., 2015). Conversely, the lactic acid bacteria grow rapidly and quickly become dominant microorganism in most cases of ensilation process(Weinberg et al., 2010). Similarly in this study, the counting of *L.palntarum* strains showed significant increase in LBM inoculated group than control. Specifically, the LAB count increased from matured late flowering stage to booting stage (28.7-63.7×10^6). This indicates higher nutrient availability of rye harvest at early stages and the possibility of

Table 2. Effect of Harvesting date and inoculation of lactic acid bacteria on pH and organic acids of rye silage

Treatment	Inoculation	pH	Lactic acid (%/DM[2)])	Acetic acid (%/DM)	Butyric acid (%/DM)	Flieg's grade
Booting Stage	Control	4.94^{ab}	1.04^{b}	0.47	0.13	good
	LBM[1)]	3.69	5.33^{a}	0.41	0.00	Excellent
Heading stage	Control	6.07^{a}	0.01^{b}	0.31	0.06	poor
	LBM	3.71^{b}	4.86^{a}	0.44	0.00	Excellent
Flowering stage	Control	6.24^{a}	0.01^{b}	0.54	0.13	poor
	LBM	3.72^{b}	4.11^{a}	0.25	0.00	Excellent
Late-flowering stage	Control	5.85^{a}	0.09^{b}	0.32	0.15	poor
	LBM	4.21^{b}	2.60^{a}	0.40	0.00	Excellent

[1)]LBM: Lactic acid bacteria mixture[2)]DM: Dry matter'

[a,b] Means with different letters within a column are significantly different at the 5% level.

Table 3. Effect of Harvesting date and inoculation of lactic acid bacteria on microbes of rye silage

Treatment	Inoculation	LAB[2)] (×10^6CFU[3)]/g)	Yeast (×10^3CFU/g)	Fungi (×10^2CFU/g)
Booting Stage	Control	5.3^{b}	7.7	-
	LBM[1)]	63.7^{a}	3.3	-
Heading stage	Control	6.0^{b}	8.7	-
	LBM	32.3^{a}	1.7	-
Flowering stage	Control	5.3^{b}	6.3	-
	LBM	27.0^{a}	2.3	-
Lateflowering stage	Control	14.6^{b}	2.7	-
	LBM	28.7^{a}	2.7	-

[1)]LBM: Lactic acid bacteria mixture,[2)]LAB: Lactic acid bacteria, [3)]CFU: Colony forming unit, [a,b] Means with different letters within a column are significantly different at the 5% level.

preservation of the higher nutrients by LAB fermentation (Muck, 2010). Further the dominant *L.plamtarum* population in silages inhibit the growth of yeast and molds that destroys the quality of the silage by reducing the pH of the silage below 5 (Li et al., 2016). Likewise, lowest yeast count and no mold count were observed in rye silage treated with LBM mixture.

Ⅳ. CONCLUSION

In conclusion, the quality of rye silage based on chemical composition and fiber degradation nature rapidly reduced as advancing maturity. Hence this study suggests that ensilling rye harvested at early stage could contain higher nutrient properties than later harvest stages by fermentation of LBM inoculants.

Ⅴ. ACKNOWLEDGEMENTS

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