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Research Article

Addition of Novel *Lactobacillus plantarum* KCC-10 and KCC-19 to Improve Fermentation Quality and Characterization of Italian Ryegrass Silage

Srisesharam Srigopalram[1], Soundharrajan Ilavenil[1], Mayakrishnan Vijayakumar[1], Hyung Soo Park[1], Kyung Dong Lee[2] and Ki Choon Choi[1]*

[1]Grassland and forage division, National Institute of Animal Science, RDA, Seonghwan-Eup, Cheonan-Si, Chungnam, 330-801, Republic of Korea, [2]Department of Oriental Medicine Materials, Dongsin University, Naju, Republic of Korea

ABSTRACT

An investigation was carried out to evaluate the potential role of *Lactobacillus Plantarum* KCC-10 and KCC-19 on the quality and fermentation characterization of Italian ryegrass (IRG) silages. The physiochemical properties of IRG silage such as crude protein content, neutral detergent fiber, acid detergent fiber, total digestible nutrient and *in vitro* dry matter digestibility were not affected by KCC-10 and KCC-19. The pH of IRG silage in KCC-10 and KCC-19 treatments decreased compared to the control ($p<0.05$), while the lactic acid content in KCC-10 and KCC-19 treatments increased compared to the control ($p<0.05$). In addition, the number of lactic acid bacteria (LAB) in the KCC-10 treatment increased compared to the control ($p<0.05$). The number of lactic acid bacteria in KCC-19 increased, but there was no significant difference in all treatments. Therefore, we recommend *L. plantarum* KCC-10 and KCC-19 as potential additive candidates in IRG silage with lots of advantages.

(**Key words :** Italian ryegrass, *Lactobacillus plantarum,* Silage, Lactic acid, Quality)

Ⅰ. INTRODUCTION

Ensiling is the process used for preserving forage crops (Wilkins et al., 1999), which known as silage. Silage confides on the amount of sufficient organic acid production for the inhibition of unwanted microbial growth under anaerobic condition (Imai, 2000). Now a day, silages are commonly preserved forages in many countries including Korea, Japan and China. In this process, the water-soluble carbohydrates are fermented into organic acids predominantly lactic acid by homo and hetrofermentative LAB (Filya et al., 2007). Forage silages stored with lactic acid bacteria (LAB) is a significant factor in determining the quality of the silage. The selection of potent lactic acid bacterial strain in national and international research is being done actively (Ely et al., 1981; Stokes, 1992). The genus *Lactobacillus* is gram positive and plays important role in the dairy industry as potential food quality maintainer and fermenting agent of dairy products (Cai et al., 1999). Fermentation by lactic acid known as homolytic fermentation is the enviable type than other fermentation while the recoveries of dry matter and energy are higher (Cao et al., 2010). *Lactobacillus* strains have been used as a starter in the preservation of dairy products, vegetables, and fish for decades. The organic acids which produced by LAB responsible for reducing pH (Weinberg et al., 2002) and inhibition of the activities of plant enzymes and pathogenic or spoilage bacteria that could decrease the nutritive values of the silages (Weinberg and Muck, 1996). LAB in the silage distribution is a potential factor in interpreting the silage fermentation characteristics. Especially the distribution of fungi in silage preparation decrease the silage quality (Weinberg et al., 2002; Filya 2003a,b). Further, fungi are the factor that results in the cattle disease because the toxins produced by secondary metabolites of fungi. Natural populations of *Lactobacillus* strains on plant materials especially forage crops are often low in number. Hence, Ensilation has the way for adding fast-growing homo fermentative *Lactobacillus* using microbial inoculation to dominate the fermentation and thereby producing higher

* Corresponding author : Ki Choon Choi, Grassland and Forage Division, National Institute of Animal Science RDA, Seonghwan-Eup, Cheonan-Si, Chungnam, 330-801 Republic of Korea.
Tel : +82-41-580-6752, Fax : +82-41-580-6779, E-mail : choiwh@korea.kr

quality silage (O'Brien et al., 2007). The bacterial strains such as *Lactobacillus Plantarum, L. acidophilus, L. fermentum, L. brevis, L. rhamnosus, L. reuterin, Enterococcus faecium and Pediococcus acidilactica* are commonly used in the homo fermentative process and available in commercial inoculants as single strain or the combination of strains (Nishino et al., 2003).

Silages from IRG, rye, whole crop barely, whole crop rice etc. are commonly used to feed livestock in Korea (Ilavenil et al., 2014). Even though high protein content of the above silages, there is some difficulty to ensile with good quality because of their low fermentable carbohydrate content, complete removal of air during ensiling by tubular hollow stems and high buffering capacity (Saarisalo et al., 2007; Muck et al., 2007). These obstacles during ensiling would be overcome by using *Lactobacillus* inoculants with possible chemicals and enzymes and the preservation of IRG, corn, rice and barley silages could be enhanced simultaneously. It is evident that the pure culture of *Lactobacillus plantarum* could not be used as silage inoculants because of its poor cell growth and fermentation metabolite production. Hence, *L. plantarum* could be used along with other species. However *L. plantarum* is the commonly found natural microflora of fermented foods, it could be used as a starter or adjacent culture, in improving organoleptic quality of the final products (Moon, 1983; Tyrolova and Vyborna, 2008). Recently, Valan Arasu et al. (2014a) and Valan Arasu et al. (2013) report that *L. plantarum* KCC-10 and KCC-19 isolated from forages interestingly showed best antifungal and probiotic effects. Therefore, the objective of this study was to evaluate the potential role of *L. plantarum* KCC-10 and KCC-19 on quality and fermentation characteristics of IRG silages.

Ⅱ. MATERIALS AND METHODS

1. Collection and preparation of silage

IRG was harvested at the early flowering stage at live stock farm, National Institute of Animal Science- RDA, Cheonan. Initially, two hundred grams of IRG were packed in an air-diffusible bag which served as control and three set of two hundred grams of IRG weighed and was packed in three different air diffusible bags. Subsequently, silage was manufactured with the addition of fermentative additive containing *L. plantarum* KCC-10 and KCC-19. Each *L. plantarum* (1.5×10^{10}cfu/g) was dissolved by distilled water (0.004 g/10ml) and inoculated with the 200 g of silage. Silage was sealed to prevent air flow. 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. Fresh culture of KCC-10 and KCC-19 were inoculated in mass cultivation medium containing glucose 1%, soy peptone 0.25%, yeast extract 1%, $MgSO_4$ 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. The powder form of KCC-10 and KCC-19 was used in silage preparation.

2. Nutrient composition and analysis

The content of crude protein was quantified by standard procedure (AOAC, 1990). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed according to the methods described by (Van Soest et al., 1993). Total digestible nutrient (TDN) was calculated as follow; 88.9-(ADF% × 0.79) (Holland et all., 1990; Seo et al., 2010). Samples were ground and passed through a 1 mm sieve prior to analysis. The *in-vitro* dry matter digestibility (IVDMD) was analyzed by the modified method of Moore (1970).

3. Enumeration of microbial populations

Ten grams of wet silage samples were transferred into sterile flasks containing 90 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). For enumeration 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 enumerated on 3 M petrifilm (3 M Microbiology Products, St.Paul, USA), and following aerobic incubation at 28 ± 1℃ for four days. Fungi were

enumerated on 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.

4. Analyzes of fermentation metabolites

10 g of sample was weighed and blended with 90 ml of deionized water for 24 hr in a refrigerator at 4℃. Firstly, 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).

5. 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 Duncan's multiple range tests were used to determine the effect of inoculants on the quality of IRG silage. Tests were run at the 5% probability level.

Ⅲ. RESULTS AND DISCUSSION

In the present study, the consequence of *L. plantarum* KCC-10 and KCC-19 on improving the quality of IRG silage was observed. By analyzing the parameters such as fermentative acids, nutritive values and microbial counts of IRG silages will give the initiative of silage quality. Organic acid producing microorganisms such as *Lactobacillus* and other *Bacillus* species have been isolated from the rumen, silages and fermented foods (Giraffa et al., 2010). Generally silages from IRG, rye and alfalfa have been fed to the live stocks (Cooke et al., 2009) in South Korea and other countries. Now a day much interest is given for the preparation of silage using IRG, barley, rye with legumes because of its low cost compared to concentrated feed, but the nutritive values have been varied by the preparation technique. On the other hand, the addition of probiotic strain such as *L. palntarum* in the silage enhances the nutritive value of the silage and this is known as ensiling method (Avila et al., 2010).

The nutritive values of IRG silage using *L. plantarum* KCC-10 and KCC-19 as inoculants have been presented in Table 1. There was a slight increase in CP level observed in *L. palntarum* KCC-10 and KCC-19 inoculated silage compared to control. Hence, it is well known that ruminal protein degradation increases with increasing CP content in the diets (Olmos Colmenero and Broderick, 2006), but addition of inoculants like homofermentative LAB can lead to the reduction of protein breakdown in silage (Merry et al., 2000). Similarly, the slight difference was noted in the ADF content between LAB applied silages and control silage. The NDF, TDN and IVDMD contents of silages were consistent, ranging from 59.12~59.52%, 67.19~67.82%, and 58.40~59.13% respectively. Therefore, no significant changes were noted for NDF, TDN and IVDMD among the treatments (Table 1).

Table 1. Nutritive value of Italian ryegrass silage according to inoculation of lactic acid bacteria

Treatment	CP[2] (%)	ADF[3] (%)	NDF[4] (%)	TDN[5] (%)	IVDMD[6] (%)
Control	10.17	37.68	59.14	67.20	59.13
LAB[1] KCC-10	10.31	38.08	59.52	67.82	58.82
LAB KCC-19	10.75	38.61	59.12	67.19	58.40

[1] LAB: lactic acid bacteria, [2] CP: Crude protein, [3] ADF: Acid detergent fiber, [4] NDF: Neutral detergent fiber, [5] TDN: Total digestible nutrient, [6] IVDMD: *in vitro* dry matter digestibility.

The number of microbial colonies in the IRG silages has been presented in Table 2. The IRG alone arbitrated group silage exhibited ($p<0.05$) lower number (104×10^7 CFU/g) of LAB compared to KCC-10 (208×10^7 CFU/g) and KCC-19 (166×10^7 CFU/g). There was a probable difference in LAB colony noted between KCC-10 and KCC-19 inoculated silages. Additionally, the number of yeast colonies in the experimental groups showed potent growth in KCC-10 supplemented silages. Further, no fungal growth was noted in the both control and experimental group and this accommodate the possible upgrade of silage fermentation with the major recovery of dry matter (Jatkauskas and Vrotniakiene, 2004).

Changes in pH and organic acids in IRG silage with KCC-10 and KCC-19 is given in Table 3. Most of the silages are susceptible to aerobic deterioration during warm climates (Danner et al., 2003). This may due to the activity of aerobic yeast that is more active in the temperature range between 25~30°C (Ashbell et al., 2002). This problem could be solved by the addition of *L. plantarum* as inoculants (Danner et al., 2003). Accumulation of homofermentative LAB will increase the lactic acid and acetic acid production and thus reducing the pH of the medium. Similarly, the amount of lactic acid produced in the *L. plantarum* KCC-10 and KCC-19 supplemented silages were higher ($p<0.05$) than the control silage. Especially, *L. plantarum* KCC-19 supplemented silage showed a significant difference compared to the control. Further, the pH of the LAB supplemented silages were lower than the control silage, particularly *L. plantarum* KCC-19 applied silage had lowest ($p<0.05$) pH. Additionally, there is notable production of acetic acid and very little butyric acid in the LAB inoculated silage with full Flieg's score which plays a significant role in the aerobic stability of silage (Filya et al., 2002). This favors improving silage fermentation and the reduction of less significant end products such as ammonia as well as volatile fatty acid, which can cause poor feed conversion and high dry matter loss (Avila et al., 2012). Hence, simultaneous production of lactic and acetic acid jointly with less residual butyric acid is potential factor for aerobic stability in silages (Ilavenil et al., 2014). Hence, supplementation of homo fermentative *L. plantarum* KCC-10 and KCC-19 with the IRG silage could be a cost-effective method for producing high-quality silage to meet the feed demand of live stocks in the growing countries.

Table 2. Changes of microbes on Italian ryegrass silage according to inoculation of lactic acid bacteria

Treatment	LAB ($\times10^7$CFU[2)]/gram)	Yeast ($\times10^4$CFU/gram)	Fungi ($\times10^4$CFU/gram)
Control	104.00[b]	14.50	0.00
LAB[1)] KCC-10	208.00[a]	16.50	0.00
LAB KCC-19	166.00[ab]	8.50	0.00

[1)] LAB: lactic acid bacteria, [2)] CFU: Colony forming 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 Italian ryegrass silage according to inoculation of lactic acid bacteria

Treatment	pH	Lactic acid (DM[2)]%)	Acetic acid (DM%)	Butyric acid (DM%)	Flieg's score
Control	4.09[a]	7.39[c]	1.10[a]	0.74[a]	78
LAB[1)] KCC-10	3.85[ab]	8.35[b]	1.07[a]	0.52[ab]	100
LAB KCC-19	3.71[b]	9.27[a]	0.48[b]	0.19[b]	100

[1)] LAB: Lactic acid bacteria, [2)] DM: Dry matter.
[a and b]: Means with different letters within a column are significantly different at the 5% level.

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