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Research Article

Lactic Acid Bacteria Mixture as Inoculants on Low Moisture Italian Ryegrass Silage Fermentation

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ABSTRACT

The effects of lactic acid bacteria (LAB) mixtures on low moisture Italian ryegrass (IRG) silage fermentation was evaluated in field conditions. The experiment was categorized into two groups: Un-inoculated (Control) and Inoculated with LAB mixture for four storage periods (45, 90, 180, and 365 days, respectively). Silage inoculated with the LAB mixture had the lowest pH with highest lactic acid production than the control from beginning at 45-365 days at all moistures. Higher LAB counts were observed in inoculated silages than the control silages at whole experimental periods. It is a key reason for the rapid acidification and higher lactic acid production in silages during the storage periods. Overall results suggest that an adding of LAB mixture had positive effects on the increasing aerobic stability of silage and preserved its quality for an extended duration.

(**Key words**: Lactic acid bacteria, Low moisture, Italian ryegrass, Fermentation)

Ⅰ. INTRODUCTION

Italian ryegrass (*Lolium multiflorum* Lam) is classified as annual ryegrass and perennial ryegrass with similar genomes (Warnke et al., 2004). They are widely distributed in North Africa, Europe, and temperate Asia (Han et al., 2013; Pan et al., 2017). In Korea, Italian ryegrass (IRG) is the most representative forage crop being cultivated more in Korea under the climate condition(Kim, 2012). Generally, IRG sowed in autumn and mostly harvested in May(Hides et al., 2006; Seo, 2005). IRG has been considered as the most potential forage crop in whole countries because of its excellent palatability, high crude protein and digestibility nutrient contents (Shehzad et al., 2014). It is high valuable forage to feed develop livestock. In recent, the utilization of IRG has been gradually increased due to its rapid growth. Therefore suitability of IRG would be increased until 2050, and then would be decreased in 2080 in relatively large numbers of regions due to a higher temperature(Kim et al., 2014). IRG swards establish quickly, the forage cut early and well suited for ensiling(Dietl and Lehmann, 2004; Succi G, 1992). Ensiling is an essential process to provide an alternative way to preserve forage grass for a long time. It makes a suitable environment for beneficial microbial growth which enhances anaerobic fermentation of existing carbohydrates in the grass. It is a great way to produce high-quality silage/ haylage for livestock's operations.

The conversion of forages into silages is an essential source of nutrients for livestock's in worldwide(Wilkinson, 2003) because it enables crops to be available for the use of whole years or restricted seasonal availability of pasture for the grazing animals. The preservation of nutrients commences with harvesting and ends when the feed is consumed by the animal. Lactic acid bacteria (LAB) have been considered as key additives that increase aerobic stability. LAB could improve the quality of silage by increasing lactic acid and decreasing pH of the silage/haylage (Arasu et al., 2014; Ilavenil et al., 2017). Inoculation of the mixture containing the heterofermentative and homofermentative lactic acid bacteria (LAB) had been successfully improved the aerobic stability and standard of fermentation of wilted grass silage(Driehuis et al., 2001). In general inoculations of heterofermentative and homofermentative or mixtures have resulted in a lower concentration of water-soluble carbohydrates (WSC) in silage (Driehuis et al., 2001; Kleinschmit and Kung, 2006).

In general, silage has a moisture content of more than 40 %. However, haylage has a moisture content of between 35 to

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60 % (wet haylage containing between 50 and 60%, dry haylage containing between 35 and 50%). Recently, Korean livestock farmers are highly interesting in haylage manufacture, even though they were used high moisture silage as forage for ruminants.

In this study, we analyzed the impact of LAB mixtures on low moistures Italian ryegrass fermentation in field condition. Organic acid profiles, microbial population and nutrient profiles of fermented silages have also been analyzed at different periods.

Ⅱ. MATERIALS AND METHODS

1. Collection of Italian ryegrass (IRG) and silage preparations

Heading stage of Italian ryegrass 'Kowinearly'(IRG) was harvested from grassland and forage farm at National Institute of Animal Science, Seonghwan-eup, Cheonan and let to wilt in field condition. After reaching expected moistures, IRG samples were ensiled with LAB mixtures (2g/tone of forage; Top haylage private limited, Jungnong Bio Inc, South Korea,) using round baler wrapping machine and kept at field condition (Arasu et al., 2014). Lactic acid bacteria were diluted in sterile distilled water. Lactic acid bacteria mixtures contain three different strains (*L. plantarum* KCC-10, K46 and KCC-19). The forage samples were wrapped six times with high quality plastic cover. Each group consists of four replicates. Organic acid profiles, microbial population and nutrient profiles were analyzed at different time intervals (45, 99, 180 and 365 days). Acid Detergent Fiber (ADF) and Neutral Detergent Fiber (NDF) contents of samples were analyzed by the method of Goering and Van Soest (1970). Total digestible nutrient (TDN) was calculated by the following formula 88.9-(ADF% × 0.79). According to AOAC (1990), crude protein (CP) content was quantified. Organic acids in the samples were quantified(Arasu et al., 2014).

2. Detection of microbial profiles

Samples (10g) were transferred into 90mL water in a conical flask and kept in an orbital shaker (Vision Scientific Co Ltd, Daejeon-Si, Korea) for 60 min at 150g. Then tenfold serial dilution was made with sterile distilled water. Bacteria in samples were counted using Quantom Microbial Cell counter (Logos biosystem, USA). In brief, one micro liter quantum total cell staining, one microliter total cell staining enhancer and ten microliters diluted samples were mixed well and kept at room temperature for 30 min. Eight microliters of cell loading buffer were then added to mixtures and mixed thoroughly without bubbles. Six microliters prepared sample was loaded onto quantum M50 cell counting slide and centrifuged at 300g for 10 minutes and then bacteria were counted with a quantum with the light intensity level set to 5 for most bacterial cells.

3. Statistical analyses

All the numerical data were obtained from three independent experiments and these data analysis was carried out using SPSS software (SPSS-16 Inc., Chicago, IL, USA). The obtained silage results were evaluated by t-test analysis and significant was set at p value less than 0.05.

Ⅲ. RESULTS AND DISCUSSION

Several tools were developed to preserve the silage/ haylage for extended storage (Borreani et al., 2018). The main goal of all scientists in the silage making is to maintain the original quality of the preserved crop as much as possible. Many additives have been used for several decades to improve the fermentation process through increasing the lactic acid content, which is a key product (Arasu et al., 2014; Kung, 2001; Kung et al., 2018; McDonald, 1981). Hence, we concentrated on the role of inoculants containing a cultured LAB, which are used to enhance the rate and extent of fermentation via increasing the lactic acid content of silage at different moistures and different periods. Here we used inoculants mixtures which consist of different *Lactobacillus plantarum*, most commonly used bacteria as an additive for silage fermentation because of its lactic acid production (Kung, 2003).

In current study, the changes of the nutritive values such as ADF, NDF TDN and CP were analyzed in LAB inoculated and non-inoculated silages. ADF, NDF TDN and CP contents of silages at 45, 90, 180, and 365 days of storage were unaffected by the addition of LAB at al moistures (Table 1). It confirmed that the addition of LAB was unaffected the native form of plant nutrients during storage, because low

Table 1. The contents of moistures and nutrient values in Italian ryegrass (IRG) silages for different storage periods

Storage periods (days)	Groups	Moisture and Nutrient values (%) in low moisture IRG silage									
		Moistures	CP[2]	ADF[3]	NDF[5]	TDN[6]	Moistures	CP	ADF	NDF	TDN
45	Control	35.6	14.4	38.0	61.9	58.9	50.1	14.8	37.3	62.0	59.4
	LAB[1)]	36.6	14.4	38.1	61.9	58.8	51.6	14.3	37.0	62.4	59.7
90	Control	37.3	14.3	37.8	61.9	59.1	49.1	14.6	37.7	62.1	59.1
	LAB[1)]	34.3	14.2	38.8	61.7	58.2	49.8	14.4	38.4	61.9	58.6
180	Control	30.5	15.0	36.5	60.6	60.1	49.2	14.1	37.5	61.9	59.3
	LAB[1)]	32.2	14.1	38.3	62.6	58.7	51.2	15.0	37.3	61.8	59.4
365	Control	32.6	13.8	37.0	61.2	59.7	51.2	14.7	36.4	60.8	60.1
	LAB[1)]	33.5	14.7	37.9	60.8	59.0	52.1	14.3	36.8	60.8	59.8

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

moisture IRG silage was normally fermented by LAB and stored. The LAB counts in experimental silages were presented in Table 2. Higher LAB counts were noted in inoculated silages in all storage periods than the controls indicated the LAB was competitive among the epiphytic microorganism. LAB counts in inoculated silages at mean moisture level 34% were 19.17 x 10^7, 11.77 x 10^7, and 6.72 x 10^7 CFU/g at 45, 90, and 180days, respectively. LAB counts in mean moisture level 50% were 21.37 x 10^7, 12.4 x 10^7, and 5.77 x 10^7, CFU/g in inoculums treated silages at 45, 90, and 180 days respectively. The population of LAB peaked at 45days (19.17 x 10^7 and 21.37 x 10^7) in both moistures condition. Lowest LAB counts were observed in control silages at all moistures throughout experimental periods (Table 2). The LAB population subsequently declined after 45 days (*P<0.05*). The decreases of LAB due to long storage periods were expected because of low pH and lack of fermentable substrates result in the death of bacteria(McDonald, 1981; Pahlow, 2003).

Similarly, LAB counts were declined in control and inoculated silages when the storage periods were extended. However, LAB was found higher in inoculated silages than the control silages at all experimental periods except 365 days. Unfortunately, we did not analyze the LAB population at 365days due to damages in the silage bales.

The decrease of pH is closely related to the conservation of the ensiled silages. The rapid acidification promotes a decrease in the enzyme-mediated proteolytic activity of the plant itself and prevents undesirable microbial growth(Davies 2005). In our study, the acidification of silages by epiphytic bacterial fermentation in the control silages reached higher pH after 45, 90, 180, and 365 days indicated that low natural bacterial counts and it has poor efficiency to initiate fermentation and control undesirable microbial growth while pH of the inoculated silages was continuously decreased at 45, 90 and 180 days whereas, pH at 365 days was not influenced by LAB. Higher acidification was noted in silages treated with LAB at 45and 90days in all moistures condition ($p<0.05$). After this peak, the pH of inoculated silages was increased until 365 days. At the same time, the LAB could maintain low pH than the control silages (Table 2).

Lactic acid, acetic acid and butyric acid are the main and dominated organic acids in fermented silages(Kung, 2001). In general, lactic acid concentration of silages increased during the ensiling process, and it lead to decreases of pH of silage (Kung et al., 2018). Lower lactic acid production in control silages with all moisture contents at all experimental periods reflected the lower LAB counts and their ability to dominate the fermentation as discussed early. Lactic acid level (4.14% and 5.35%) was peaked at day 90 in 34% and 50% moistures, respectively than the untreated silages. The concentration of lactic acid was reduced after 90 days ($P<0.05$).

Either high or low lactic acid productions were observed in LAB treated silages throughout periods whereas lactic acid level was not detected in untreated silages after 180 days. There were differences in concentration of acetic acid and butyric acid between untreated and LAB treated silage, butyric acid content was noted only at day 180 in 34% moisture silage, rest of the days butyric acid level was not detected in

Table 2. LAB population, pH and organic acids level in Italian ryegrass (IRG) silages for different storage periods

Storage periods (days)	Groups	Lactic acid bacteria and organic acids level (%) low moisture IRG silage											
		LAB[1]	pH	Lactate[2]	Acetate[2]	Butyrate[2]	Flieg's score	LAB	pH	Lactate	acetate	butyrate	Flieg's score
45	Control	6.6	5.3[a]	0.0[b]	0.3	0.0	55	11.3[b]	4.59[a]	2.17[b]	0.30	0.34[a]	72
	LAB[2)]	19.1	4.38[b]	2.7[ba]	0.2	0.0	100	21.3[a]	3.85[b]	4.78[a]	0.31	0.04[b]	100
90	Control	3.83	5.18[a]	0.1[b]	0.4	0.0	57	6.3[b]	4.95[a]	2.09[b]	0.55[a]	0.24[a]	78
	LAB[2)]	11.7	4.14[b]	4.2[a]	0.3	0.0	100	12.4[a]	3.95[b]	5.35[a]	0.31[b]	0.06[b]	100
180	Control	2.2	5.62[a]	0.0[b]	0.1	0.3[b]	25	4.3[b]	5.02[a]	0.00[b]	0.27	0.24	13
	LAB[2)]	5.3	4.75[b]	1.3[a]	0.2	0.6[a]	57	5.8[a]	4.30[b]	2.96[a]	0.31	0.31	73
365	Control	-	5.58[a]	0.0[a]	0.2[b]	0.0	50	-	5.36[a]	0.00[b]	0.00	0.00	50
	LAB[2)]	-	5.26[b]	1.0[b]	1.4[a]	0.0	61	-	5.16[b]	0.70[a]	0.00	0.03	49

[1]LAB: Lactic acid bacteria 10^7CFU/gram; [2] Percentage of organic acids
a and b: Means with different letters within a column are significantly different at the 5% level.

both control and LAB treated silages.

In contrast, the butyric acid level was detected in 50% moisture samples throughout experimental periods, became more evident, starting at 45 days of ensiling and continued to 365 days (Table 2). The simultaneous production of lactic acid and acetic acid with less amount of butyric acid is important factors that can reach aerobic stability of silages(Danner et al., 2003). The content of acetic acid and butyric acid are primary negative indicators of the quality of the silage fermentation process and also it affects dry matter and energy content during fermentation (Pahlow et al., 2003, Eisner et al., 2006). Lower concentration of acetic acid in LAB mixture inoculated silages at 90dayse was observed in 50% moisture whereas other low moisture silages inoculated with LAB exhibited higher acetic acid level at 360 days.

Similarly, this study also confirmed that the addition of LAB to silage improving its quality by reducing the pH of the silages by organic acid productions during fermentation. The butyric acid concentration was lower at an early storage period in 50% moisture. Later periods of storage, acetic acid and the butyric acid level was slightly increased or unchanged between control and LAB inoculated in both moistures. As we discussed early the decreases in LAB counts over extended periods was expected due to low pH and lack of fermentable substrates available in silages as results in the death of LAB and may start to grow enterobacteria, its second most numerous bacterial group of the epiphytic microbiota active in the silo. Silage quality is closely associated with enterobacteria population and its rate of decline because these microorganisms are main competitors with LAB for available of sugars that make gas loss and loss of nutritional content of silages(Muck, 2010).

As shown in the above Flieg's score study, there was no difference between LAB inoculated and non-inoculated silages in the storage days 180 and 365 days. After six months, it was found that the organic acid in the silage might be vaporized/ degraded due to damage in plastic bags which used to cover the silage bale and it permits oxygen level in samples that favor undesirable microbial growth and spoilage. Therefore, this study suggest that it is necessary to increase the number of plastic wraps more than six times for long time silage storage with high quality.

Ⅳ. CONCLUSION

Ensiling of silages with LAB mixtures strongly improved quality of silages at different moistures level and extended storage periods. LAB had positive effects on enhancing the quality silage particularly enhanced the production of lactic acid and reduced pH of the silages, which prevents the entophytic bacterial growth during storage. Also, adding LAB was not affected the native form of plant nutrients. Overall data were suggesting that addition of LAB mixture as additives during low moisture IRG silage manufacture by the method of ensiling with high-quality biodegradable plastic prevent aerobic deterioration silage for a long time.

Ⅴ. ACKNOWLEDGEMENT

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