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Research Article

Effect of Selected Inoculant Applications on Chemical Compositions and Fermentation Characteristics of High Moisture Rye Silage

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ABSTRACT

The aim of this study was to investigate the effect of isolated lactic acid bacteria (LAB) on the quality of high moisture rye silage. Rye forage (*Secale cereale* L.) was harvested at the heading stage (27.3% of dry matter (DM)) and cut into approximately 3-5 cm lengths. Then, the forage divided into 4 treatments with different inoculants: 1) No additives (**CON**); 2) *Lactobacillus brevis* strain 100D8 at a 1.2 x 10^5 colony-forming unit (cfu)/g of fresh forage (**LBR**); 3) *Leuconostoc holzapfelii* strain 5H4 at a 1.0 x 10^5 cfu/g of fresh forage (**LHO**); and 4) Mixture of LBR and LHO (1:1 ratio) applied at a 1.0 x 10^5 cfu/g of fresh forage (**MIX**). About 3 kg of forage from each treatment was ensiled into a 20 L mini-bucket silo in quadruplicate for 100 days. After silo opening, silage was collected for analyses of chemical compositions, *in vitro* nutrient digestibilities, fermentation characteristics, and microbial enumerations. The CON silage had the highest concentrations of neutral detergent fiber and acid detergent fiber ($p = 0.006$; $p = 0.008$) and a lowest *in vitro* DM digestibility ($p < 0.001$). The pH was highest in CON silage, while lowest in LBR and MIX silages ($p < 0.001$). The concentrations of ammonia-N, lactate, and acetate were highest in LBR silage ($p = 0.008$; $p < 0.001$; $p < 0.001$). Propionate and butyrate concentrations were highest in CON silage ($p = 0.004$; $p < 0.001$). The LAB and yeast counts were higher in CON and LHO silages compare to LBR and MIX silages ($p < 0.001$). However, the mold did not detect in all treatments. Therefore, this study could conclude that *L. brevis* 100D8 and *Leu. holzapfelii* strain 5H4 can improve the digestibility and anti-fungal activity of high moisture rye silage.

(Key words: Antifungal, Digestibility, Lactic acid bacteria, Silage fermentation)

Ⅰ. INTRODUCTION

High moisture forages could decrease a silage quality and produce a great amount of silage extraction (Vetter and Von Glan, 1978). In addition, high moisture silage could lead to occur the abnormal fermentation by the growth of undesirable microbes, which could initiate high productions of butyrate and ammonia-N (Leibensperger and Pitt, 1987). Wilting process and absorbent application to meet the ideal moisture content of forages (approximately 65%) are the well-known approaches to ensure the better quality of silages (McDonald et al., 1991). For these reasons, the moisture of forages is recommended to regulate through wilting process. However, in some cases, forage wilting is difficult to apply due to weather and limitation of facility in the field.

Undesirable microbes, such as yeast, mold, or clostridia leads to increase nutrient loss during ensiling (McDonald et al., 1991). In high moisture content, those microbes can grow rapidly and increase the concentrations of ammonia-N and butyrate, which inhibit the decrease of pH and then reduce the quality of silage. In addition, butyrate is also commonly found in high moisture silage as the results of clostridia fermentation (McDonald et al., 1991). The presence of butyrate during ensiling presented disadvantage effect on silage, which reduce the palatability and acceptability for animals (McDonald et al., 1991; Nkosi and Meeske, 2010). Inoculation of lactic acid bacteria can help to reduce the negative effects of high moisture content of forage during the ensiling period (Paradhipta et al., 2019). The hetero-

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fermentative lactic acid bacteria (LAB) can be recommended as inoculant for high moisture silage due to their ability to produce organic acid with high antifungal activity, such as acetate and propionate (Paradhipta et al., 2019). Previous study was reported that those organic acids were effective to inhibit undesirable microbes in silage (Adesogan et al., 2004; Danner et al., 2003).

Rye (*Secale cereale* L.) is one of the important winter forages for ruminants in South Korea (Kim et al., 1986; Lee et al., 2021a). However, the use of rye as a silage still has a limitation especially with the wilting process due to the cold weather or rain period during harvesting. In addition, rye is usually harvested at the heading stage to increase dry matter (DM) yield, but it could decrease fermentation quality and nutrient digestibility due to the high moisture content. The study of specific LAB for rye silage is necessary to improve the nutrient digestibility and fermentation quality. Recently, previous studies isolated new strains of LAB from rye silage and selected based on fibrinolytic and antifungal activities (Kim et al., 2017; Kim et al., 2018), consisting of *Leuconostoc holzapfelii* strain 5H4 and *Lactobacillus brevis* strain 100D8. *Leu. holzapfelii* strain 5H4 was reported to produce esterase, cellulase, and xylanase (Kim et al., 2017), while *L. brevis* strain 100D8 produced lanthionine as antifungal substance (Kim et al., 2018). In our previous studies, those new LABs on the rye silages resulted the improvement of fermentation characteristics and nutrient digestibility (Paradhipta et al., 2020; Lee et al., 2021a, 2021b). However, those new LABs did not test for the high moisture of rye silage, which is harvested at the heading stage.

Therefore, this research was conducted to determine the effects of isolated LAB on the fermentation quality and *in vitro* nutrient digestibility of high moisture rye silage, which was harvested at the heading stage.

Ⅱ. MATERIALS AND METHODS

1. Silage production

Rye (*Secale cereale* L.) forage was grown in the animal research unit, Gyeongsang National University, Jinju, South Korea, and harvested at the heading stage. The DM content of harvested rye forage was 27.3%, approximately. The harvested forage was cut into approximately 3-5 cm lengths and divided into 4 treatments with different inoculants: 1) No additives (CON); 2) *L. brevis* strain 100D8 at a 1.2×10^5 colony-forming unit (cfu)/g of fresh forage (LBR); 3) *Leu. holzapfelii* strain 5H4 at a 1.0×10^5 cfu/g of fresh forage (LHO); and 4) Mixture of LBR and LHO (1:1 ratio) applied at 1.0×10^5 cfu/g of fresh forage (MIX). Each treatment was sub-sampled (1 kg) promptly for analysis of nutrient contents. Following this, about 3 kg of forage from each treatment was ensiled into a 20 L bucket silo with 4 replications for 100 days.

2. Chemical compositions and *in vitro* nutrient digestibility

Ten grams of the sub-sampled rye forage and silage was dried at 105°C for 24 h to measure DM content. Another 200 g (approximately) of each silage sub-sample was then collected, dried at 60°C for 48 h, ground, and sieved through a 1 mm screen using a cutting mill (SHINMYUNG ELECTRIC Co., Ltd, South Korea). The crude ash content (CA) was determined using a muffle furnace at 550°C for 5 h. Crude protein (CP) and ether extract (EE) contents were analyzed by the Kjeldahl method (method 984.13) and the Soxhlet method (method 920.39), respectively. Neutral detergent fiber (NDF; method 2002.04) and acid detergent fiber (ADF; method 973.18) contents were established using an Ankom^{200} fiber analyzer (Ankom Technology, Macedon, NY, USA). All protocols for CP, EE, NDF, and ADF analyses were described by AOAC (2005). Hemicellulose level was ascertained by calculating the difference between NDF and ADF. *In vitro* digestibility of DM (IVDMD) and NDF (IVNDFD) were determined using the method of Tilley and Terry (1963) using Ankom^{II} Daisy Incubators (Ankom Technology, Macedon, NY, USA).

3. Fermentation characteristics

Twenty grams of rye forage and silage were blended with 200 mL of distilled water for 30 s, and then filtered through two layers of cheesecloth for pH, lactate, and VFA analysis. Silage pH was measured using a pH meter (SevenEasy, Mettler Toledo, Greifensee, Switzerland), and ammonia-N was determined by the colorimetric method following the protocol of Chaney and Marbach (1962). Silage extract was centrifuged at 5645 ×g for 15 min, and HPLC (L-2200; Hitachi, Tokyo, Japan) fitted with a UV detector (L-2400; Hitachi) and a column (Metacarb

87H; Varian, CA, USA) was used to measure the concentrations of lactate and VFA as described by Adesogan et al. (2004).

4. Microbial enumerations

About 20 g of silage from each treatment was diluted with 180 mL of distilled water and macerated in a blender to obtain silage extract for enumeration of LAB, yeast, and mold. Considering the silage extract as the first dilution, serial dilutions were prepared and 100 μL aliquots of three consecutive dilutions (10^{-4}, 10^{-5}, and 10^{-6}) were plated in triplicate onto a selective agar medium. Lactobacilli MRS agar media (MRS; Difco, Detroit, MI, USA) was used to culture LAB, and potato dextrose agar (PDA; Difco, Detroit, MI, USA) was used for yeast and mold. The MRS agar plates were placed in a CO_2 incubator (Thermo Scientific, USA) at 30°C for 72 h, while PDA plates were incubated at 30°C for 72 h in a standard incubator (Johnsam Corporation, Korea). Visible colonies were counted from the plates, and the number of cfu per gram of silage was expressed.

5. Statistical analysis

All collected data in the present study were statistically analyzed as a completely randomized design by ANOVA procedure of the statistical analysis system (SAS), package program, version 9.3 (SAS, 2011). Its general model was Yij = μ + Ti + eij, where Yij = response variable, μ = overall mean, Ti = the effect of inoculant, eij = error term. Mean separation was performed using Tukey's test. Significant differences were declared at $p < 0.05$.

Ⅲ. RESULTS

The chemical compositions and *in vitro* nutrient digestibility of rye forages harvested at the heading stage were shown in Table 1. The mean concentrations of CP, NDF, ADF, IVDMD, and IVNDFD of rye forages were 9.91, 64.9, 40.1, 64.4, and 51.5%, respectively.

The chemical compositions and *in vitro* nutrient digestibility of rye silages ensiled for 100 days were shown in Table 2. All contents had no inoculant effects except to NDF, ADF, and IVDMD. The LBR, LHO, and MIX silages had a lower concentration of NDF than CON silages ($p = 0.006$; 67.0, 67.3, and 67.0 vs. 69.0%), and LBR silages had a lowest concentration of ADF ($p = 0.008$). On other hand, IVDMD was higher in LHO and MIX silages than in CON and MIX silages ($p < 0.001$; 67.4 and 68.0 vs. 60.7 and 62.5%).

The fermentation characteristics of rye silages ensiled for 100 days were shown in Table 3. The pH was lowest ($p < 0.001$) in LBR and MIX silages, but highest in CON silage. The concentrations of ammonia-N, total organic acid, and lactate were highest in LBR silage ($p = 0.008$; $p < 0.001$; $p < 0.001$). Acetate concentration in LBR and MIX silages were higher than that in CON and LHO silages ($p < 0.001$; 5.90 and 4.96 vs. 2.97 and 2.66%), while propionate and butyrate concentrations were highest ($p = 0.004$; $p < 0.001$). Lactate to acetate ratio was highest in LHO silage, but lowest in CON

Table 1. Chemical compositions and *in vitro* digestibility of rye forage harvested at the heading stage (% DM)

	Treatment			
	CON	LBR	LHO	MIX
Dry matter	27.5	26.8	26.5	28.4
Crude protein	9.43	9.88	9.94	10.4
Ether extract	2.47	2.48	2.44	2.55
Crude ash	7.99	7.76	8.59	7.97
Neutral detergent fiber	65.8	64.9	64.4	64.4
Acid detergent fiber	39.2	40.9	40.6	39.5
IVDMD	63.8	64.3	64.4	65.1
IVNDFD	50.8	50.4	51.7	53.1

CON, distilled water (2 mL/kg); LBR, *Lactobacillus brevis* 100D8 (1.2×10^5 cfu/g); LHO, *Leuconostoc holzapfelii* 5H4 (1.0×10^5 cfu/g); MIX, mixture of LBR and LHO at 1:1 ratio; IVDMD, *in vitro* DM digestibility; IVNDFD, *in vitro* NDF digestibility.

Table 2. The effects of selected inoculant applications on chemical compositions and in vitro digestibility of rye silage harvested at the heading stage and ensiled for 100 days (% DM)

	Treatment				SEM	*p*-value
	CON	LBR	LHO	MIX		
Dry matter	23.2	23.8	23.0	23.7	0.565	0.305
Crude protein	9.96	10.2	9.83	10.1	0.189	0.102
Ether extract	4.51	4.84	4.61	4.88	0.413	0.610
Crude ash	9.35	9.33	8.75	8.63	0.318	0.323
Neutral detergent fiber	69.0^{a}	67.0^{b}	67.3^{b}	67.0^{b}	0.749	0.006
Acid detergent fiber	43.4^{a}	41.6^{b}	42.6^{ab}	42.2^{ab}	0.590	0.008
IVDMD	60.7^{b}	62.5^{b}	67.4^{a}	68.0^{a}	0.917	<.001
IVNDFD	51.3	51.0	51.5	52.6	1.369	0.445

CON, distilled water (2 ml/kg); LBR, *Lactobacillus brevis* 100D8 (1.2×10^{5} cfu/g); LHO, *Leuconostoc holzapfelii* 5H4 (1.0×10^{5} cfu/g); MIX, mixture of LBR and LHO at 1:1 ratio; IVDMD, *in vitro* DM digestibility; IVNDFD, *in vitro* NDF digestibility; SEM, standard error of the mean.
$^{a, b}$ Means in the same row with different superscripts differ significantly ($p < 0.05$).

Table 3. Effect of selected inoculant applications on fermentation characteristics of rye silage harvested at the heading stage and ensiled for 100 days (% DM)

	Treatment				SEM	*p*-value
	CON	LBR	LHO	MIX		
pH	5.04^{a}	4.26^{c}	4.51^{b}	4.26^{c}	0.091	<.001
Ammonia-N	0.09^{b}	0.13^{a}	0.10^{b}	0.09^{b}	0.007	0.008
Total organic acid	14.6^{b}	18.1^{a}	10.7^{c}	12.4^{bc}	1.004	<.001
Lactate	0.50^{c}	7.04^{a}	4.74^{b}	4.29^{b}	0.866	<.001
Acetate	2.97^{b}	5.90^{a}	2.66^{b}	4.96^{a}	0.525	<.001
Propionate	6.57^{a}	5.15^{ab}	3.27^{b}	3.13^{b}	1.011	0.004
Butyrate	4.58^{a}	ND^{b}	ND^{b}	ND^{b}	0.912	<.001
Lactate:acetate ratio	0.17^{c}	1.19^{ab}	1.78^{a}	0.86^{b}	0.201	<.001

CON, distilled water (2 ml/kg); LBR, *Lactobacillus brevis* 100D8 (1.2×10^{5} cfu/g); LHO, *Leuconostoc holzapfelii* 5H4 (1.0×10^{5} cfu/g); MIX, mixture of LBR and LHO at 1:1 ratio; SEM, standard error of the mean; ND, < 0.01% DM.
$^{a\sim c}$ Means in the same row with different superscripts differ significantly ($p < 0.05$).

Table 4. Effects of selected inoculant applications on microbial counts of rye silage harvested at the heading stage and ensiled for 100 days (log10 cfu/g)

	Treatment				SEM	p-value
	CON	LBR	LHO	MIX		
Lactic acid bacteria	6.99^{a}	4.95^{b}	6.51^{a}	5.31^{b}	0.274	<.001
Yeast	6.41^{a}	5.25^{b}	6.71^{a}	5.59^{b}	0.181	<.001
Mold	ND	ND	ND	ND	-	N/A

CON, distilled water (2 ml/kg); LBR, *Lactobacillus brevis* 100D8 (1.2×10^{5} cfu/g); LHO, *Leuconostoc holzapfelii* 5H4 (1.0×10^{5} cfu/g); MIX, mixture of LBR and LHO at 1:1 ratio; SEM, standard error of the mean; ND, < 4.0 log10 cfu/g; N/A, not available.
$^{a, b}$ Means in the same row with different superscripts differ significantly ($p < 0.05$).

silage ($p < 0.001$; 1.78 vs. 0.17).

The microbial counts of rye silages ensiled for 100 days are shown in Table 4. The LAB count was higher in CON and LHO silages than LBR and MIX silages ($p < 0.001$; 6.99 and 6.51 vs. 4.95 and 5.31 log10 cfu/g), while yeast count was lower in LBR and MIX silages than in CON and LHO silage

($p < 0.001$; 5.25 and 5.59 vs. 6.41 and 6.71 log10 cfu/g). However, the mold did not detect by 4.0 log10 cfu/g in all treatments.

Ⅳ. DISCUSSION

In previous studies, the concentrations of CP, NDF, and ADF in rye forage were 6.4 - 10.6, 61.2 - 68.2, and 37.7 - 40.4%, respectively (Han et al., 2015; Kim et al., 2015; Choi et al., 2016). In the present study, the concentrations of CP, NDF, and ADF in the rye forage were 9.91, 64.9, and 40.1%, which were in normal ranges compared to those previous studies. Kang et al. (2009) and Xie et al. (2021) reported that some LAB producing fibrinolytic enzymes can increase the digestion of ruminants by degrading fiber complex consisting of cellulose, hemicellulose, or lignocellulose. Especially for lignocellulose, the use of ferulic-acid esterase can set a free of cellulose and hemicellulose from lignin complex linkage (Adesogan et al., 2014). In addition, Nsereko et al. (2008) demonstrated that the increase of forage fibrinolysis by inoculant indicates a positive correlation with the rumen microbial activity. *Leu. holzapfelii* 5H4 used in the present study (LHO and MIX silages) also was known that has fibrinolytic enzymes such as cellulase, xylanase, and esterase (Kim et al., 2017), and its ability was demonstrated by Paradhipta et al. (2020). The present study also had shown increased IVDMD in LHO and MIX silages, and it might be resulted by the application of *Leu. holzapfelii* 5H4 which can produce fibrinolytic enzymes.

Lactate has a higher acidification value than another organic acids to decrease silage pH (Aksu et al., 2004). In the present study, the lactate concentrations of LBR, LHO, and MIX silages were higher than CON silage, and led to low pH in LBR, LHO, and MIX silages than CON silage. Hartinger et al. (2019) reported that the final pH should be 4.5 or less to product high quality silage without abnormal fermentation. In the present study, the pH silages applied LAB were about 4.5 or less. However, the pH of CON silage was 5.04 by low lactate concentration, which was higher than suggested optimal fermentation pH of silage, and it means that the silage could be occurred abnormal fermentation. According to Yang (2000), the pKa values of acetate and propionate are 4.75 and 4.87 respectively. It indicates that acetate and propionate also affect the decrease of silage pH. In the present study, the difference in pH between LHO and MIX silages could be supported by the difference in acetate concentration of those silages. Generally, VFAs such as acetate and propionate were known to have anti-fungal activity (Woolford, 1975; Danner et al., 2003), but the presence of these VFAs is not always positive in silage. Kung and Shaver (2001) reported that VFAs could be increased by undesirable bacteria which can lead to abnormal fermentation. The present of butyrate is avoided in silage due to indicating clostridia fermentation (Kung et al., 2018; Leibensperger and Pitt, 1987; McDonald et al., 1991). In the present study, butyrate was only detected in CON silage, and it indicates that the silage might be contaminated by undesirable bacteria such as clostridia. The reason of no butyrate in LBR, LHO, MIX silages might be due to the application of hetero-fermentative LAB (*L. brevis* strain 100D8 and *Leuc. Holzapfelii* strain 5H4), which can stimulate the optimum fermentation rapidly. Tlowest lactate to acetate ration of CON silage in the present study could be supported by higher propionate and butyrate concentrations in CON silage.

Yeast, which is classified as a member of the fungal species, can increase the silage pH by converting lactic acid into CO_2, H_2O, and heat and lead to the growth of various other undesirable bacteria in silage (McDonald et al., 1991). *L. brevis* 100D8 used in the LBR and MIX inoculants was investigated that inhibit the growth of yeast or mold by antifungal and acidify activity (Kim et al., 2018). Acetate produced from hetero-fermentative LAB, such as *L. brevis* 100D8 can inhibit the growth of undesired bacteria such as yeast and mold by respiratory inhibition through structural damage in the cell (Kang et al., 2003). Propionate was known that can inhibit the growth of undesired bacteria by mitochondria apoptosis of the cell (Yun and Lee, 2016). In addition, *L. brevis* 100D8 was known that has genes encoding lanthionine synthetase C-like protein (Kim et al., 2018), which can inhibit the growth of toxic fungal such as *Aspergillus*, *Penicillium*, and *Fusarium* species (Paul and Donk, 2005). Lee et al. (2021a) demonstrated that silages applied *L. brevis* 100D8 were inhibited the growth of yeast and increased aerobic stability. In the present study, LBR and MIX silage had lower yeast count with higher acetate and propionate by *L. brevis* 100D8 application and it was similar with the result of Lee et al. (2021b).

Ⅴ. CONCLUSION

The LBO and LBR silages were improved IVDMD and inhibited the growth of yeast, respectively. Especially, MIX silage applied the mixture of LBR and LHO inoculants at a 1:1 ratio was improved both IVDMD and anti-fungal ability of rye silage harvested at the heading stage in the present study. In conclusion, *L. brevis* 100D8 and *Leu. holzapfelii* strain 5H4 were confirmed that can improve DM digestibility and anti-fungal activity of rye silage harvested at the heading stage and ensiled with high moisture content.

Ⅵ. ACKNOWLEDGMENT

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