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Research Article

Effects of Lactic Acid Bacteria, Storage Temperature and Period on Fermentation Characteristics, and *in vitro* Ruminal Digestibility of a Total Mixed Ration

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ABSTRACT

This study evaluated the effect of lactic acid bacteria (LAB, a mixture of *Enterococcus faecium* and *Lactobacillus plantarum*) supplementation, the storage temperature, and storage period on the fermentation characteristics and *in vitro* ruminal digestibility of a total mixed ration (TMR). The TMR was prepared into two groups, namely, CON (control TMR without the LAB) and ML (supplementing a mixture of *E. faecium* and *L. plantarum* in the ratio of 1% and 2% (v/w), respectively). Both groups were divided and stored at 4°C or 25°C for 3, 7, and 14 d fermentation periods. Supplementing LAB to the TMR did not affect the chemical composition of TMR except for the lactate and acetate concentration. Storage temperatures affected ($p<0.05$) the chemical composition of the TMR, including pH, lactate, and acetate contents. The chemical composition of TMR was also affected ($p<0.05$) by the storage period. During *in vitro* rumen fermentation study, the ML treatment showed lower ($p<0.05$) dry matter digestibility at 24 h incubation with a higher pH compared to the CON. There was no difference in the *in vitro* dry matter digestibility (IVDMD) of TMR between the CON and ML treatment however, at 24 h, ML treatment showed lower ($p<0.05$) IVDMD with a higher pH compared to the CON. The effects of storage temperature and period on IVDMD were not apparent at 24 h incubation. In an *in vivo* study using Holstein steers, supplementing LAB to the basal TMR for 60 d did not differ in the final body weight and average daily gain. Likewise, the fecal microbiota did not differ between CON and ML. However, the TMR used for the present study did include a commercial yeast in CON, whereas ML did not; therefore, results were, to some extent, compromised in examining the effect of LAB. In conclusion, storage temperature and period significantly affected the TMR quality, increasing acetate and lactate concentration. However, the actual effects of LAB supplementation were equivocal.

(Key words: Lactic acid bacteria, Storage period, Storage temperature, Total mixed ration)

Ⅰ. INTRODUCTION

Total mixed ration (TMR) is a mixture of forage, concentrates, and other micronutrients formulated appropriately to provide the nutrient requirements of ruminants (Wang et al., 2010). Using TMR, it is possible to reduce feed costs without compromising productivity using agricultural or food by-products which are known to be rich in some nutrients (Kim et al., 2020). Furthermore, feeding TMR to ruminants can prevent the feed sorting behavior by ruminants (especially with a high moisture TMR), induce the balanced intake of forage and concentrates, stabilize rumen fermentation, and improve the feed intake and the efficiency of nutrients utilization (Felton and DeVries, 2010; Bueno et al., 2020). Therefore, TMR is a popular feeding regime in numerous beef and dairy farms in Korea. However, due to limited production and supply of home-grown forages other than rice straw, farmers depend on imported forages, and hence, low-priced agricultural by-products are often used as feed ingredients for TMR (Choi et al., 2006; Choi et al., 2012; Song et al., 2020). Moreover, TMR prepared using agricultural by-products usually had a high moisture content, where molds develop rapidly and affect TMR quality (Ki et al., 2007). With the distinctive four seasons in Korea, the TMR quality is likely to differ heavily based on the season. Cao et al. (2011) reported that the fermentation quality and microbial differences were affected by seasons, especially in

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cold regions. Therefore, appropriate storage in terms of time and temperature is essential.

Recently, in the production of TMR, microorganisms have been used to improve the fermentation quality of TMR. Among them, the inoculation of lactic acid bacteria (LAB) rapidly reduces the pH during fermentation and the loss of dry matter (Nishino et al., 2004). In addition, LAB have been proven to maintain aerobic stability while increasing lactic acid production and preventing spoilage (Weinberg and Muck, 1996). As stated above, several studies have examined the effect of various lactic acid bacteria as feed additives, but studies with *Enterococcus faecium* as feed additives in Korea are rare. Hanchi et al. (2018) mentioned that *E. faecium* has the potential to be used as probiotics. Pang et al. (2014) reported that the addition of gram-positive *E. faecium* to feed increased the *in vitro* rumen digestibility and speed up the fermentation, increasing volatile fatty acids (VFAs) concentration. On the other hand, *Lactobacillus plantarum* has been proven to increase the silage and TMR fermentation quality (Ki et al., 2007; Yuan et al., 2016; Marbun et al., 2020; Xie et al., 2020). Nevertheless, studies with optimum conditions for example, temperature or period of storage following TMR manufacturing, is limited although there were some studies in relation to a storage period or time when silage was prepared (Muck and Dickerson, 1988; Wang and Nishino, 2008; Liu et al., 2011). Therefore, we hypothesized that the interactions among these factors (i.e., LAB, storage period, and temperature) have potential to increase the quality of TMR.

Therefore, this study aimed to evaluate the effect of LAB (*E. faecium* and *L. plantarum*) supplementation, storage periods, and temperatures 1) on the fermentation characteristics of TMR and 2) on *in vitro* ruminal digestibility. In addition, the effect of LAB supplementation was examined further on animal performance using Holstein steers.

Ⅱ. MATERIALS AND METHODS

1. Total mixed ration preparation

Experimental TMR was prepared either with (ML) or without (CON) the mixture of two different lactic acid bacteria. According to storage temperature, each TMR was divided and stored at 4°C and 25°C. Additionally, to investigate the effect on the fermentation period, sampling on day 3, 7, and 14 from the day of TMR preparation were performed. Hence, total of 12 treatments with 3 factors (LAB, storage temperature and period) were prepared in triplicate.

The total mixed ration originating from Anseong TMR feed (Anseong, Gyeonggi-do) was used in the CON group. Meanwhile, for the ML group, the brewer's yeast was omitted from the CON group TMR, and lactic acid bacteria in a liquid medium developed by Genebiotech Co., Ltd. (Gongju-si, Republic of Korea) was inoculated. A total of 52 kg of fresh TMR was divided equally into CON and ML groups. In order to equalize the moisture content up to 45%, an aliquot of 9.4 kg of sterile distilled water was added to the CON, and 8.62 kg of sterile distilled water mixed with 2 types of lactic acid bacteria (*E. faecium* (1.0×10^7 cfu/g) and *L. plantarum* (2.0×10^7 cfu/g)) in volumes of 260 mL and 520 mL, respectively, were added to the ML group. Then, one kg of each experimental TMR was vacuum-packed, stored in a refrigerator at 4°C or an incubator at 25°C, and fermented for 3, 7, and 14 days. The CON and ML groups were prepared in triplicate.

2. Sample collection and chemical analysis

After each ensiling period, the vacuum-packed samples were opened. Aliquot 100 g of TMR was dried at 60°C for 3 days to remove the excess moisture. The dried samples were ground using an ultra-centrifugal mill ZM 200 (Retch, Germany) and were determined for dry matter (DM), organic matter (OM), crude protein (CP) and ether extract (EE) according to AOAC (2019) procedures. In addition, neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed according to Van Soest et al. (1991).

The rest of the fresh samples were stored at -20°C for later being thawed before analysis. Aliquot 100 g of the thawed frozen sample and 900 mL of distilled water were placed in a zipper bag and homogenized using a stomacher (Bag Mixer®, Interscience, France) for four min. The supernatant was used to determine the pH and organic acid profile. The supernatant was filtered using a 0.45 μm syringe filter (Rephile, RjN1345NH, China) and analyzed using high-performance liquid chromatography (HPLC, Prostar, Varian, USA) equipped with s SUPELCOGEL C-610H (30 cm × 7.8 mm diameter) column (Supelco, Sigma-

Aldrich, USA) and UV/VIS detector (210 nm) for determining the lactate concentration. The column temperature was set at 30°C, and the mobile phase was 0.1% phosphoric acid (H_2PO_4) at a flow-rate of 0.5 mL/min (Ryu et al., 2017).

3. *In vitro* rumen fermentation study

The *in vitro* rumen fermentation experiment was performed according to Tilley and Terry (1963) method. The rumen fluid was collected from two Hanwoo cattle fed with rice straw and a commercial concentrate located at the Kyungpook National University Sangju campus experimental farm. The collected rumen fluid was placed in a thermos bottle and brought to the laboratory within 30 min, filtered using 8 layers of muslin under CO_2 flow. Aliquot 1.8 L of filtered rumen fluid was diluted with McDougall's buffer (McDougall, 1948) on 1:4 ratio. The 50 mL diluted buffer-rumen fluid was dispensed into 165 mL-culture bottles containing 0.5 g of dried and ground TMR according to treatments and incubated at 39°C for 3 and 24 h. Each treatment was duplicated. After incubation, pH and dry matter digestibility (DMD) were determined. The serum bottle was opened, and the contents were filtered using a 5 × 10 cm nylon bag (ANKOM Technology, USA, pore size 50 μm). After filtration, the contents remaining in the nylon bag were placed in a 105°C drying oven, dried for 24 h and weigh for determining DMD, while the supernatant was used for measuring pH of the supernatant.

4. Animal study, fecal collection and microbial analysis

A feeding study was conducted on 30 Holstein steers (461.8 ± 73.39 kg BW) divided into two groups, CON and ML. A pen with a size of 5 × 10 m^2 was occupied by 5 cattle and triplicated for each treatment. The CON group was fed by conventional TMR (Anseong TMR, Anseong-si, Gyeonggi-do), while for the ML group TMR, *L. plantarum* (2.0×10^7 cfu/g) and *E. faecium* (1.0×10^7 cfu/g) were added to CON group TMR on 1% addition level (w/v). In addition, brewer yeast was added instead of LAB for the CON group. Both TMR moisture levels were set up to 38% and fermented for 7 d at the ambient temperature. The ingredient of TMR used in this study was presented in Table 1. Experimental feed was fed twice a day for 60 d. Water was provided *ad libitum*. The body weights of animals were measured every 30 d after morning feeding at the same time two times. Daily weight gain was calculated from the difference in body weight between the early and end of the study divided by the experimental period in days.

Denaturing gradient gel electrophoresis (DGGE) analysis was performed to evaluate the effect of the LAB supplementation in the TMR on the microbial population in the gastrointestinal tract using feces. Three animals were randomly selected from each of the CON and ML groups for manual fecal collection during weighing. The collected fecal samples were immediately stored in an icebox, moved to the laboratory, and stored in a -20℃ freezer until analysis. For analyzing the changes in rumen-derived microorganisms of fecal samples, genomic DNA was extracted according to Rius et al. (2012) and amplified using 341F-GC (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG) CAG CAG) and 534R (ATT ACC GCG GCT GCT GG) primers. The PCR conditions were with denaturation at 94℃ for 5 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, and extension at 72°C for 30 sec, and terminated with a final extension at 72°C for 7 min. Gel electrophoresis was performed to check the amplification results using 2% of agarose gel electrophoresis. The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Germany) and continued with DGGE using a D-code system (Bio-Rad, USA). The 8% (w/v) polyacrylamide gel, containing 20% to 80% denaturant gradient in 1 × TAE buffer, was used. The electrophoresis was operated at 130 V for 8 h. For gel staining, ethidium bromide (EtBr, Bio-Rad, USA) was put in 250 mL running buffer for 20 min. The stained gels were visualized under UV light using Gel-Doc XR+ (Bio-Rad, USA). Subsequent analysis was performed using the SYStat13 program.

The viability of bacteria *(Salmonella sp*., *Escherichia coli*, and LAB) was measured using the method conducted by Dave and Shah (1996). The aliquot 1 g sample was 10-fold serially diluted (10^3 to 10^7) using saline water. The solutions were spread around the MRS agar (Difco, Detroit, MI, USA) using the spread-plate technique and incubated at 37°C for 48 h. The enumeration was recorded on the plate containing colony forming unit (CFU) between 30 to 300.

5. Statistical analysis

The analysis of variance (ANOVA) was conducted to examine the effects of LAB, storage temperatures, periods, and

Table 1. Feed ingredients of total mixed ration used in this study (As-fed basis, %)

Ingredients	Treatments	
	CON[1]	ML
Basal concentrates	6.0	6.0
Oat hull	13.0	13.0
Corn gluten feed	4.5	4.5
Flaked corn	7.5	7.5
Sesame oil meal	7.6	7.6
Beet pulp	2.0	2.0
Oat	2.0	2.0
Dried corn husk	3.0	3.1
White pan bread	2.0	2.0
Brewer's grains	24.0	24.0
Soy sauce cake	5.5	5.5
Yeast for brewing	7.0	-
Lactic acid bacteria	-	3.0
Water	-	4.0
Cotton seed	4.0	4.0
Vitamin mix	0.4	0.4
Sodium bicarbonate	0.4	0.4
Limestone	0.4	0.4
Salt	0.1	0.1
Timothy hay	2.0	2.0
Italian ryegrass	1.0	1.0
Mixed hay	7.5	7.5
Total	100.0	100.0

[1]CON: TMR without lactic acid bacteria (LAB), ML: TMR supplemented with LAB.

their interactions. In addition, the results of dry matter intake and daily gain of Holstein steers were analyzed by t-test. The significance level was declared at $p<0.05$. The statistical analysis was performed using SPSS software (Version 25, IBM, USA).

Ⅲ. RESULTS AND DISCUSSION

This study aimed to examine the effect of LAB supplementation, storage temperature, and period on the nutritive values of a TMR. However, we realized a problem in terms of diet formulation where the CON, the basal TMR, included yeast, but the ML (supplemented with LAB) did not include yeast, as presented in Table 1. Such a problem was noticed when the experiment was completed. It is speculated that the effect of supplementing LAB in the TMR was likely to be compromised by the effect of yeast in the CON. Therefore, extended discussion on LAB supplementation seems to be complicated. Nevertheless, it is hoped that discussion on storage temperature and period may still be of value to provide additional information to the animal industry.

1. Changes in the chemical composition of TMR

The effect of LAB, storage periods, and temperatures on the chemical composition of TMR is presented in Table 2. The DM contents were shown to be significantly ($p<0.05$) affected by the storage periods and also temperatures. The storage temperature also affected ($p<0.05$) the DM, OM, CP, and EE content. The addition of LAB to TMR reduced crude protein content of the TMR ($p<0.05$). Storage periods also showed a significant effect ($p<0.05$) on DM, OM, EE, NDF, and ADF contents. The interaction of storage periods × temperatures

influenced the CP and EE content (p<0.05).

The pH of TMR was significantly affected by the storage temperatures (Table 3). Storing TMR at 4°C had shown a higher (p<0.05) pH compared to 25°C. On the other hand, the lactate and acetate were influenced (p<0.05) by adding LAB, storage periods, and temperatures. The lactate content was higher (p<0.05) in the ML at 25°C storage temperature of all storage periods compared with those of 4°C. The interaction of LAB × storage temperatures and storage periods × temperatures significantly (p<0.05) affected the lactate concentration.

In this study, the inoculation of LAB did not affect the chemical composition of the TMR except for CP. However, the study by Marbun et al. (2020) on maize silage reported a contrary result compared to this study. It showed that *L. plantarum* inoculation had higher CP contents than the control. A study by Nkosi and Meeske (2010) also showed contrary results with this study which mentioned that inoculation of LAB to TMR could elevate DM compared to the other group. Such a difference might be due to the short fermentation or storage period of the TMR. For example, the studies above fermented the TMR for 90 d. However, the study by Nishino et al. (2004) presented that DM loss did not happen after 10 d of inoculation of LAB to TMR, which was similar to our results.

The TMR with LAB (ML) showed lower (p<0.05) CP content than the CON. The reason might be that the protein

Table 2. Effect of lactic acid bacteria, storage periods, and temperatures on the chemical composition (% of dry matter) of total mixed ration

Variable	CON[1]						ML						SEM[2]	p-value[3]		
	3 days		7 days		14 days		3 days		7 days		14 days			L	D	T
	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C				
DM[4]	46.88	45.11	46.89	45.15	46.26	44.81	46.08	45.64	45.71	45.66	45.05	44.90	0.109	0.128	0.037	0.000
OM[5]	93.90	93.80	94.04	93.88	94.30	94.48	94.01	93.85	94.19	94.01	94.73	94.81	0.101	0.641	0.000	0.006
CP[6]	17.50	17.84	17.08	18.03	17.32	17.74	16.07	16.63	16.42	17.84	17.07	16.73	0.076	0.000	0.217	0.001
EE[7]	6.90	7.16	8.46	9.81	7.96	9.47	7.31	7.47	8.24	10.44	8.12	8.64	0.118	0.993	0.000	0.001
NDF	45.60	48.58	46.73	44.90	46.78	45.51	50.22	49.37	47.29	45.39	47.92	46.56	0.363	0.061	0.037	0.353
ADF[8]	25.57	26.82	26.09	25.19	26.10	25.54	29.31	28.63	25.93	25.06	26.71	25.00	0.246	0.083	0.005	0.250

[1]CON: TMR without lactic acid bacteria (LAB), ML: TMR supplemented with LAB.
[2]SEM: standard error of the mean.
[3]L: effect of LAB, D: effect of storage periods, T: effect of storage temperatures.
[4]Interaction of LAB × storage temperature was significant (p<0.05).
[5]Interaction of LAB × storage period × temperature effect was significant (p<0.05).
[6,7]Interaction of storage period × temperature effect was significant (p<0.05).
[8]Interaction of LAB × storage period was significant (p<0.05).

Table 3. Effect of lactic acid bacteria, storage periods, and temperatures on the pH, lactate, and acetate (g/kg DM) of total mixed ration

Variable	CON[1]						ML						SEM[2]	p-value[3]		
	3 days		7 days		14 days		3 days		7 days		14 days			L	D	T
	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C				
pH	5.05	4.73	5.40	4.31	5.13	4.28	5.38	4.26	5.38	4.22	5.08	4.28	0.039	0.505	0.212	0.000
Lactate[4]	38.14	69.93	36.45	89.78	37.93	87.26	46.36	74.87	44.37	88.17	54.97	78.58	1.123	0.050	0.018	0.000
Acetate[5]	2.02	5.99	2.13	8.40	4.15	12.70	2.16	5.26	2.19	7.94	3.67	9.86	0.246	0.000	0.000	0.000

[1]CON: TMR without lactic acid bacteria (LAB), ML: TMR supplemented with LAB.
[2]SEM: standard error of the mean.
[3]L: effect of LAB, D: effect of storage periods, T: effect of storage temperatures.
[4]Interaction of inoculant × storage temperature and storage period × temperature effects were significant (p<0.05).
[5]Interaction among factors (inoculant × storage period, inoculant × temperature, storage period × temperature and inoculant × storage period × temperature) effects were significant (p<0.05).

breakdown occurred to transform true protein into non-protein compounds. This phenomenon was known to be performed by plant and microbial enzymes during fermentation in the ensiling process (McDonald et al., 1991). Nevertheless, the LAB addition increased lactate and acetate concentration in TMR. This result was consistent with the report that a substantial amount of lactate was produced during fermentation due to adding lactic acid bacteria to a feed (Filya, 2003; Kim et al., 2009). In addition, Nishino et al. (2004) and Keles and Demirci (2011) reported that lactic acid bacteria produced lactate during fermentation and caused a decrease in pH. However, in this study, the pH was not affected by lactate.

Different from silage, TMR can be manufactured all year round. However, TMR manufactured has to be stored in low temperatures during winter and in high temperatures during summer (Wang and Nishino, 2013). Such difference is likely to induce changes in chemical composition, which may affect palatability and, subsequently, the dry matter intake of an animal. Due to this ambient environment, efforts to examine the effect of storage temperature on a TMR prepared are necessary. In our study, storage temperature affected the chemical composition of the TMR. The TMR stored at 25°C produced higher (p<0.05) lactate and acetate compared to the one stored at 4°C. The study by Kim et al. (2009) reported that the optimum growth temperature for lactic acid bacteria was 25°C. Therefore, in the results of this experiment, the reason for the high level of lactate in the treatment group at 25°C might be that active fermentation occurred due to the considerable growth of lactic acid bacteria at the optimal temperature and a significant amount of lactate was produced. Moreover, TMR silage produced insufficient amounts of lactic acid when stored at low temperatures (Cao et al., 2011). Wang and Nishino (2013) also stated that high temperatures could increase acetate production in TMR silage which explains higher acetate concentration in TMR stored at 25°C.

The storage period also took a remarkable effect on the TMR chemical composition. A more extended storage period decreased (p<0.05) the DM, OM, EE, and acetate concentration but increased (p<0.05) the lactate concentration. In comparison, the study by Weinberg and Chen (2013) on wheat and corn silage showed that a short storage period resulted in unstable aerobic stability. On the contrary, more extended storage could enhance DM loss. According to Weinberg and Muck (1996), the fermentation state in ensiling period usually lasts for one to two weeks (7 to 14 d). In this state, the LAB becomes the dominant microbes, producing organic acids such as lactate and acetate, and the pH decreases.

2. Ruminal digestibility and pH *in vitro*

Table 4 presents the results of DMD and pH of TMR during *in vitro* rumen fermentation. At 3 h, LAB addition did not provide any significant effect on DMD. After 24 h incubation,

Table 4. Effect of lactic acid bacteria, storage periods, and temperatures on the dry matter digestibility (DMD, %) of total mixed ration at 3 and 24 h of *in vitro* rumen fermentation technique

Variable	CON[1]						ML						SEM[2]	p-value[3]		
	3 days		7 days		14 days		3 days		7 days		14 days			L	D	T
	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C	4°C	25°C				
3 h																
DMD[4]	42.06	36.56	42.73	41.98	42.55	43.30	36.51	42.93	42.29	45.38	38.95	41.00	0.309	0.576	0.000	0.116
pH[5]	6.97	6.97	7.00	7.00	7.02	7.00	7.00	7.00	7.02	7.00	7.03	7.01	0.002	0.000	0.000	0.031
24 h																
DMD[6]	62.84	58.08	59.18	57.44	58.30	57.13	53.81	55.11	52.35	59.30	53.66	54.25	0.474	0.000	0.359	0.840
pH[7]	6.81	6.85	6.84	6.87	6.85	6.86	6.87	6.87	6.87	6.84	6.88	6.88	0.003	0.000	0.073	0.152

[1]CON: TMR without lactic acid bacteria (LAB), ML: TMR supplemented with LAB.
[2]SEM: standard error of the mean.
[3]L: effect of LAB, D: effect of storage periods, T: effect of storage temperatures.
[4]Interaction of LAB × storage period, inoculant × storage temperature and inoculant × storage period × temperature effects were significant (p<0.05).
[5]Interaction of storage period × temperature was significant (p<0.05).
[6,7]Interaction of inoculant × temperature was significant (p<0.05).

Table 5. Effect of total mixed ration treated with mixed lactic acid bacteria at ambient temperature for 7 d on the body weight gain of Holstein steers

Items	Treatments[1]		SEM[2]	*p*-value
	CON	ML		
Initial body weight (kg)	428.9	434.9	41.91	0.924
Final body weight (kg)	492.5	499.9	35.87	0.892
Total body weight gain (kg)	63.6	64.9	6.46	0.891
Daily body weight gain (kg/day)	1.06	1.08	0.11	0.886

[1]CON: TMR without lactic acid bacteria (LAB), ML: TMR supplemented with LAB.
[2]SEM: standard error of the mean.

Table 6. Effect of total mixed ration treated with mixed lactic acid bacteria at room temperature for 7 d on the changes of selected fecal microbes of Holstein steers (log cfu/g)

Variable	Treatments[1]		SEM[2]	*p*-value
	CON	ML		
Salmonella sp.	0.33	1.13	0.546	0.358
E. coli	5.53	6.07	0.342	0.332
Lactic acid bacteria	5.87	5.26	0.436	0.378

[1]CON: TMR without lactic acid bacteria (LAB), ML: TMR supplemented with LAB.
[2]SEM: standard error of the mean.

the ML had lower ($p<0.05$) DMD compared to the CON. The ML group on 14 d fermentation had lower ($p<0.05$) DMD compared to the CON. These results were contrary to the study by Pang et al. (2014), which added *E. faecium* to TMR and proved that *E. faecium* increased the DMD during *in vitro* study. The temperature did not have any significant effect on DMD. Conversely, the TMR stored for 7 d has the highest ($p<0.05$) DMD at 3 h incubation compared to other storage period groups.

3. Animal study and microbial changes

Based on the results of the chemical composition analysis of TMR and the *in vitro* study, we decided to conduct the animal experiment by combining 3 factors. We prepared the treated group TMR using inoculant, stored for 7 d at ambient temperature, and offered it to Holstein steers. The final, total, and daily body weight gain of Holstein steers were not affected by the treated TMR (Table 5) when fed for 60 d. This result was contrary to a study by Nkosi and Meeske (2010), which mentioned that TMR inoculated by LAB could significantly increase the final body weight and average daily gain of lambs when fed for 63 d.

The viable cell counts (VCC) of fecal microbes of Holstein steers were not significantly different between the CON and the ML (Table 6), although there were numerical changes between treatments. Likewise, the cluster analysis from the DGGE result showed that a shift in the microbial community in the feces of animals offered CON and ML was hardly distinguishable. Animals in the same pen (e.g., no. 8 and 9, no. 3 and 4 from CON, and no. 12 and 13, 17 and 18, 19 and 20) were clustered together, indicating that they were offered a similar diet. However, there was no apparent, distinct clustering between CON and ML.

In this study, the fermentation characteristics and digestibility of the TMR with LAB were marginal compared to the CON. We propose that such responses might be due to the use of yeast for brewing (7%, as-fed basis) in the CON-TMR, which was omitted in the preparation of the ML-TMR. Therefore, the actual effect of LAB might have been masked by omitting the yeast in the dietary formulation, which was not anticipated. Further study with the same dietary condition with or without LAB is needed to examine the actual effect of LAB on TMR. Nevertheless, findings on lactate and acetate concentration in the supplemented TMR with extended storage periods and higher temperatures may be of use for feeding TMR to ruminants.

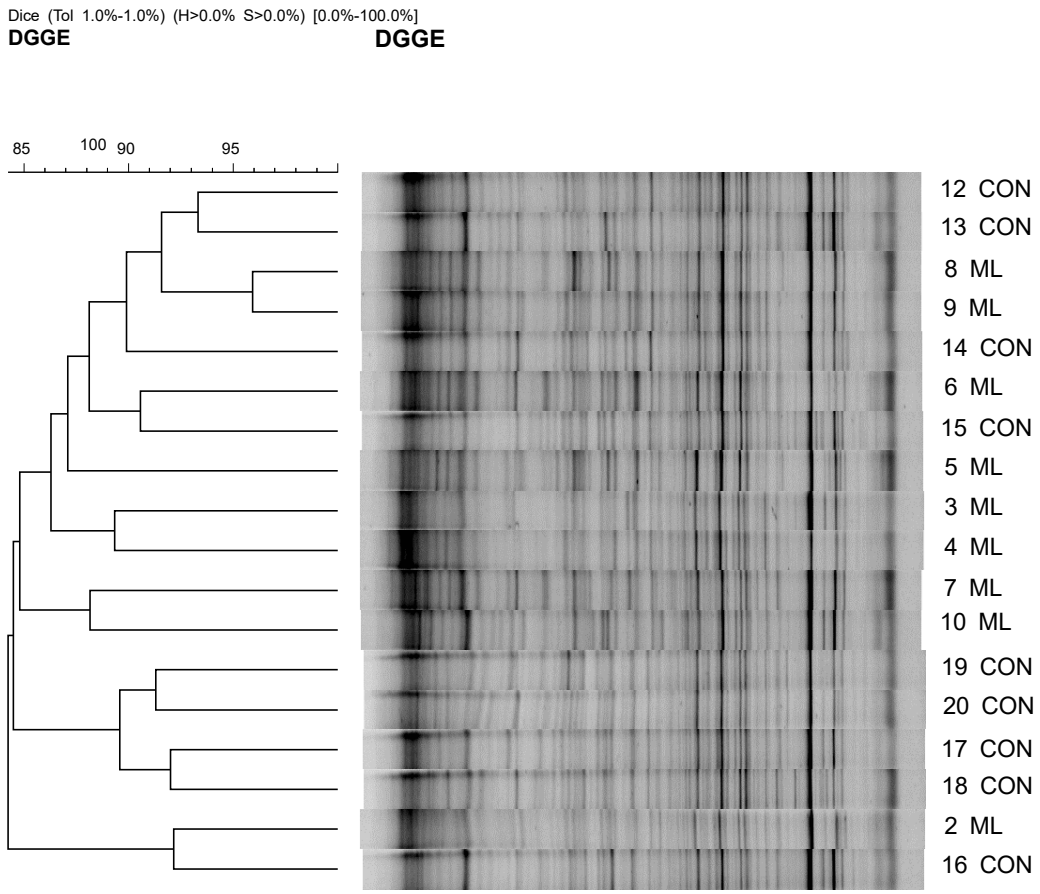

Fig. 1. Fecal bacterial communities of Holstein steers offered total mixed ration with (ML) or without (CON) lactic acid bacteria analyzed using denaturing gradient gel electrophoresis (DGGE).

Ⅳ. CONCLUSSION

The purpose of the present study was to examine the effect of LAB, storage periods, and temperatures on the chemical composition of a TMR and its *in vitro* ruminal digestibility. Further study with Holstein steers examined the effect of LAB on animal performance and fecal microbiota. Extended storage and higher temperature increased lactate and acetate concentration in both TMR. However, the effect of LAB supplementation was marginal. The effect on the microbial community and enumeration using feces from Holstein steers was not apparent. However, the TMR used for the present study did include a commercial yeast in CON, while ML did not; therefore, results were, to some extent, compromised in examining the effect of LAB. In conclusion, LAB inoculation, storage temperature, and period significantly affected the TMR quality, yet the results on LAB supplementation were equivocal. Therefore, further study is needed to examine the actual effect of LAB along with storage temperature and periods *in vitro* and *in vivo*.

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