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Research Article

Effects of Lactic Acid Bacteria Inoculant on Fermentation Quality and in vitro Rumen Fermentation of Total Mixed Ration

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ABSTRACT

Fermented total mixed ration (TMR) is a novel feed for ruminants in South Korea. The purpose of this study was to evaluate the effects of lactic acid bacteria (LAB) on the quality of TMR and *in vitro* ruminal fermentation. Strains of three LAB spp. (*Lactobacillus plantarum*, *L. brevis, L. mucosae*) were used in fermentation of TMR. Inoculations with the three LAB spp. lowered pH and increased concentrations of lactic acid, acetic acid, and total organic acid compared to non-LAB inoculated control (only addition of an equivalent amount of water) ($p<0.05$). Bacterial composition indicated that aerobic bacteria and LAB were higher. However, *E. coli* were lower in the fermented TMR than those in the control treatment ($p<0.05$). Among the treatments, *L. brevis* treatment had the highest concentration of total organic acid without fungus detection. Gas production, pH, and ammonia-nitrogen during ruminal *in vitro* incubation did not differ throughout incubation. However, ruminal total VFA concentration was higher ($p<0.05$) in the LAB spp. treatments than the control treatment at 48 hours. Overall, the use of *L. brevis* as an inoculant for fermentation of high moisture. TMR could inhibit fungi growth and promote lactic fermentation, and enhance digestion in the rumen.

(**Key words**: *In vitro* fermentation, Lactic acid bacteria, Total mixed ration)

Ⅰ. INTRODUCTION

Total mixed ration (TMR) has been widely used for lactating cow feeding and balanced with feed ingredients such as forages, grains, proteins, minerals, vitamin, and additives to meet the livestock's nutritional requirements. Total mixed ration has limitations to be used in some farms due to the requirement for labor forces and equipment. In South Korea, fermented TMR has been increasingly used for beef cattle, goat, and pig feeds (Lee et al., 2009; Cha et al., 2018). However, fermented TMR enhances preference through changes in smell and flavor, and provides stable and nutritionally balanced feed (Nishino et al., 2003; Yuan et al., 2015). In South Korea, because imported hay is used for most forage, it has a low water content, which leads to separation of forages and concentrated feed, and so water is added when making TMR. For that reason, TMR has an around 40% water content, which becomes a risk factor for spoilage either due to the state of the ingredients or due to mold and/or aerobic bacteria.

Inoculation of lactic acid bacteria (LAB) aims to induce dominance of the inoculated strain over the native bacterial population and to suppress the growth of undesirable microorganisms (Wilkinson et al., 2003; Saarisalo et al., 2007). *Lactobacillus* spp. can be used for TMR, which can improve the fermentation of TMR through lowering pH, increasing lactic acid content, and improving Frieg's mark and V-score (Cao et al., 2010). Also, inoculation with lactic fermenting microorganisms can prevent a decrease of nutrient content in TMR, compared to non-treated control during TMR production (Ki et al., 2007). According to a report by Shioya (2008), lactic acid bacteria dominant in TMR leads to low pH, high lactic acid, but low butyric acid content. It also results in an increase in dry matter (DM) intake, crude protein (CP), ether extract (EE), neutral detergent fiber (aNDF) digestion rate, and gross digestible energy compared to non-fermented TMR. Feeding with fermented TMR is effective at reducing nutritional loss because it improves digestion rate (Yang et al., 2010).

Lactobacillus spp. are subgrouped by their carbohydrate fermentation profiles, namely, obligate homo-fermentative, facultative hetero-fermentative, and obligate hetero-fermentative. Hetero-fermentative *Lactobacillus* produces lactate, acetate, and propionate to inhibit pathogenic bacteria and fungi. It has been reported to be excellent for silage stability and to increase silage digestibility of livestock (Nsereko et al. 2008). Therefore, the fermentation

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characteristics of TMR and its *in vitro* ruminal fermentation characteristics were evaluated using three LAB species.

Ⅱ. MATERIALS AND METHODS

1. Isolation, identification and metabolite production of microbes

Two animal samples, including Korean native cattle feces and Korean native goat rumen fluid were collected for isolation of LAB from Sunchon National University animal farm. Each sample was pre-cultivated at 37°C for 30 min in an incubator in a sterile tube containing 50 ml of 0.85% saline. The sample was 10-fold serially diluted in 0.85% saline, and 0.1 ml of the sample was spread on LAMVAB medium plate (Hartemink et al., 1997) and incubated at 37°C for 48h. After incubation, greenish colonies and yellow around randomly picked from the plates and transferred to MRS agar (Merck, Germany). Isolated microorganisms were genetically characterized and identified by amplifying the 16S rRNA gene sequences of using primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). PCR products were purified by using a QIAquick PCR Purification Kit (Qiagen, USA). Purified PCR products of 16S rDNA were sent to Macrogen (Macrogen, Korea) for DNA sequencing. Sequenced fragments were assembled using the SeqMan program (DNA Star, USA). The gene sequences were then compared with 16S RNA gene sequences available in EzTaxon and NCBI-BLAST programs. Isolates were re-cultivated in broth medium at 39°C for 24 h in a shaking incubator at 120 g. One ml sample of each culture was then transferred to respective Eppendorf tubes and immediately centrifuged at 16 609 g for 10 min at 4°C in a Micro 17TR centrifuge (Hanil Science Industrial Co. Ltd, Korea). The resulting supernatants were filtered through 0.2-㎛ Millipore filters, transferred to clear vials and sealed with screw caps for determination of VFA concentrations. Standards with R^2 = 0.999 were prepared prior to sample analysis. Volatile fatty acid concentrations were measured via high-performance liquid chromatography (HPLC) using a device (Agilent Technologies 1200 series) with a UV detector set at 210 and 220 nm. A MetaCarb 87H (Varian, USA) column was used in the determination of fermentation products, and a 0.0085 N H2SO4 buffer was applied to the column at a rate of 0.6 ml min^{-1} (Tabaru et al. 1988). The VFA concentration in mmol l^{-1} was calculated in ppm divided by the molecular weight.

2. Fermentation of TMR

Total mixed ration (TMR) was balanced using Italian ryegrass (IRG), corn flake (Corn), wheat bran (WB), lupin seed (LS), and concentrated feed (CF). The proportions of IRG, Corn, WB, LS, and CF were fixed at 200, 250, 200, 50 and 300 g/kg TMR dry matter (DM), respectively. TMR was prepared as follows: IRG, Corn, WB, LS, and CF were mixed and added to 10% LAB culture ($1x10^8$cfu/mL), and 30% distilled water. This TMR was then adjusted with water to a moisture content of 385g/kg. TMR mixtures were put in a plastic box and mixed to get a uniform distribution of each ingredient, and then packed into zipper bags (30cm diameter x 35cm height, Clean wrap, Korea). There were 3 replications for each treatment. The TMRs were treated with no lactobacillus inoculation (control), *L. plantarum* (T1), *L. brevis* RNAL14 (T2), *L. mucosae* 521129 (T3). Silage bags were incubated 37°C for 7 days.

3. Fermentation quality analysis

Fermentation qualities were determined by measuring pH, organic acid, chemical composition and microbial count of the TMR 5.0g fresh sample was homogenized used a reciprocating shaker for 30min with 45mL of sterilized distilled water. The pH was measured with a Pinnacle series M530p meter (Schott instruments, Germany). After centrifugation (16,609 × *g*, 10 min, 4°C), the supernatant was analyzed for organic acid concentrations. The supernatant was passed through a 0.2 μm millipore filter under pressure and then injected into a High-performance liquid chromatography (Agilent Technologies 1200 series) system to determine organic acid concentrations. HPLC with a UV detector set at 210 nm and 220 nm. A MetaCarb 87H (Varian, Germany) column was used in the determination of fermentation products with application of 0.0085 N H_2SO_4 buffer at a rate of 0.6 mL/min (Tabaru et al., 1988). The organic acid concentration in mM was calculated in ppm divided by the molecular weight. Fermented feed was analyzed for DM and CP according to the AOAC (1990). The microbiological assay of the TMR was performed by the serial

Table 1. Composition of feed ingredient and chemical composition of total mixed rations

Ingredient composition(g/kg)	
IRG†	200.00
Corn Flake	250.00
Wheat Bran	200.00
Lupin Seed	50.00
Concentrates	300.00
Chemical composition	
Dry matter	615.80
Crude Protein (g/kg DM)	129.10
Ether extract (g/kg DM)	25.98
Crude Ash (g/kg DM)	44.33
NDF (g/kg DM)	356.28
ADF (g/kg DM)	161.25

†IRG: Italian ryegrass, NDF: neutral detergent fiber, ADF: acid detergent fiber, DM: dry matter

dilution and agar plate count method. Total aerobic bacteria were enumerated using tryptic soy agar (Difco, USA). LAB was enumerated using 0.04% bromocresol purple (Sigma, USA) with Lactobacilli MRS agar (Difco). Mold growth was measured using yeast extract glucose chloramphenicol agar (Merck, USA). Coliforms were enumerated using MacConkey agar (Difco, USA).

4. *In vitro* rumen fermentation

Ruminal contents were obtained from three 48-month-old rumen-cannulated Holstein Friesian cow with body weights of 600 kg ± 47 kg that had been fed twice a day with concentrate feed (NongHyup Co, Korea) and rice straw at a 2:8 ratio. Rumen fluid collection was made by hand, after which the contents were squeezed and strained through four layers of cheesecloth. Ruminal fluid was pooled in prewarmed amber bottles covered with foil, sealed, and immediately transported to the laboratory; the temperature was maintained at 39°C. Buffer solution was prepared according to the procedure described by Asanuma et al, (1999) with the following composition in mg/L: dipotassium phosphate (K_2HPO_4), 450; monopotassium phosphate (KH_2PO_4), 450; magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 190; calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$), 120; sodium chloride (NaCl), 900; cysteine hydrochloride ($C_3H_7NO_2S \cdot HCl$), 600; ammonium sulfate ($(NH_4)_2SO_4$), 900; trypticase peptone (BBL, USA), 1000; and yeast extract (Difco, USA), 1000. The prepared medium was then autoclaved at 121°C for 15 min and maintained in a 39°C water bath and flushed with N_2. The pH of the buffer was adjusted to 6.9 using 10 N NaOH, after which the particle-free rumen fluid was added. Next, 100 mL of buffered rumen fluid was anaerobically transferred to each of the serum bottles containing the 1% DM of distinct TMR. The bottles were subsequently sealed with rubber septum stoppers and aluminum caps and incubated at 39°C for 48 h in an HB-201SF shaking incubator (Hanbaek Scientific, Korea) at 100 rpm. Experimental treatments were replicated three times for each incubation period.

5. Parameters for analysis of *in vitro* rumen fermentation

At the end of each incubation period, parameters were measured in each of the serum bottles. The pH was measured with a Pinnacle series M530p meter (Schott Instruments, Germany) immediately after uncapping each bottle. Total gas was determined using a press and sensor machine (EA-6 Laurel Electronics, USA). Briefly, a needle channel connected to the machine was extended into the sealed fermentation bottle to measure the accumulated gas pressure in the headspace of the bottle. A gas flow regulator was subsequently opened, allowing the gas to flow inside a syringe barrel. The plunger was pulled gradually until the pressure reading in the machine display reached zero and the volume of gas trapped inside the barrel was recorded as the total gas produced in ml. Additionally, 1.0 ml of fermenta from each of the serum bottles were immediately centrifuged at 16,609 × g for 10 min at 4°C using a Micro 17TR centrifuge (Hanil Science Industrial Co. Ltd., Korea). The supernatant and the pellet were separated and kept in 1.5 mL Eppendorf tubes and then frozen at -80°C until ammonia-nitrogen (NH_3-N), VFA, and molecular analyses. The NH_3-N concentration was measured according to the methods developed by Chaney and Marbach (1962) using a Libra S22 spectrophotometer (Biochrom Ltd., England) at an absorbance of 630 nm. For determination of VFA concentrations, samples in eppendorf tubes were thawed at room temperature, after which they were filtered through 0.2 μm millipore filters for determination of VFA concentrations. Standards with $R^2 = 0.999$ were prepared prior to sample analysis. VFA concentrations were measured using high-performance liquid chromatography (Agilent Technologies 1200 series) with a UV detector set at 210 nm and 220 nm. A MetaCarb 87H (Varian, Germany) column was used in the determination of fermentation

products with application of 0.0085 N H_2SO_4 buffer at a rate of 0.6 ml/min (Tabaru et al., 1988). The VFA concentration in mM was calculated in ppm divided by the molecular weight.

6. Statistical analysis

Data were analyzed by analysis of variance using the general linear model (GLM) for randomized complete block design. All treatments were conducted in triplicate and Duncan's multiple range test (DMRT) was used to identify differences between specific treatments. A P value < 0.05 indicate statistical significance. All analyses were carried out using Statistical Analysis Systems (SAS, 2004).

Ⅲ. RESULTS

1. Isolation and identification of LAB

A total of 50 bacterial strains were isolated from rumen fluid of Korean native goat and from feces of Korean native cattle. Subsequently, 16s rRNA sequencing and phylogenetic analyses demonstrated that *L. mucosae* 521129 was 99.92% similar to *L. mucosae* S32, *L. brevis* RNAL14 was 99.86% similar to *L. brevis* ATCC14869. The metabolite production profiles of isolates after batch cultivation and 24 h of continuous culturing are summarized in Table 3. *L. brevis* had concentration of lactate and acetate of 156.65 mM and 105.35 mM, respectively while *L. mucosae* had propionate concentration of 33.74 mM.

2. Fermentation quality

Fermentation characteristics, microbial components and chemical composition of TMR after 7 days of ensiling are presented in Table 3. The pH value, lactic, and acetic concentrations of fermented TMR ranged from 3.95~5.97, 7.25~40.20 mM, and 14.65~33.29 mM, respectively. pH was lowest (p<0.05) in the T1 (3.95). Meanwhile, lactic acid concentration was highest (p<0.05) in the T1 (40.20mM). The acetic acid concentration was highest (p<0.05) in the T3 (33.29 mM). Additionally, the population of aerobic bacteria and LAB was highest (p<0.05) in the T1 (7.44 and 7.75 $\log_{10}$ cfu/g) while fungi was highest in T3 (2.22 $\log_{10}$ cfu/g) but not detected in T2. The DM and CP content of fermented TMR ranged from 653.76 g/kg ~ 660.84 g/kg and 110.28 g/kg ~ 125.20 g/kg, respectively. Additives had no effect on DM content (p>0.05), but the TMR with *L. mucosae* contained significantly highest of crude protein content (p<0.05).

3. Rumen fermentation *in vitro* parameters

The effects of fermentation TMR on rumen fermentation parameters *in vitro* are presented in Table 4 and 5. The total gas production and ammonia-nitrogen concentration, for all treatments, increased along with the duration of the incubation time while pH values declined. Total gas production was highest (p<0.05) in T3 at 6 h of incubation, but no significant difference in pH was detected (p<0.05). The pH value was lower (p<0.05) in control than the treatment groups at 3 h of incubation. Ammonia nitrogen (NH_3-N) concentration was significantly higher (p<0.05) in control than the treatment groups at 0 h of incubation but there was no significant difference in

Table 2. Similarity of bacteria strains isolated from rumen fluid of native Korean goats or feces of native Korean cattle with *Lactobacillus* spp. Determined using 16S rRNA sequence analysis and their organic acid production

Strains	Similar *Lactobacillus* spp†	Organic acid(mM)		
		Lactate	Acetate	Propionate
Lactobacillus plantarum KACC11451	-	141.32	66.93	4.93
Lactobacillus brevis RNAL14	*Lactobacillus brevis* ATCC14869^T	156.65	105.35	29.70
Lactobacillus mucosae 521129	*Lactobacillus mucosae* S32^T	103.33	81.54	33.74

†Similarity was determined when similarity percentage was greater than 99%

Table 3. Fermentation characteristics of TMRs treated with lactic acid fermenting bacteria after 7 days of fermentation period

Parameters	TMR treatment†			
	Control	T1	T2	T3
Fermentation characteristics				
pH	5.97^{a}±0.01	3.95^{d}±0.02	4.11^{c}±0.04	4.64^{b}±0.07
Lactic acid (mM FM)	7.25^{d}±0.09	40.20^{a}±0.05	25.12^{b}±0.09	20.85^{c}±0.26
Acetic acid (mM FM)	15.33^{c}±0.15	14.65^{d}±0.08	29.91^{b}±0.02	33.29^{a}±0.12
Total organic acid (mM FM)	22.59^{c}±0.23	54.84^{ab}±0.05	55.03^{a}±0.10	54.13^{b}±0.36
Microbial components				
Aerobic bacteria (log_{10} cfu/g FM)	4.66^{d}±0.03	7.44^{a}±0.02	6.46^{c}±0.04	6.83^{b}±0.05
LAB (log_{10} cfu/g FM)	3.63^{d}±0.11	7.75^{a}±0.03	6.43^{c}±0.13	7.36^{b}±0.02
Fungi (log_{10} cfu/g FM)	2.22^{c}±0.16	3.00^{b}±0.00	0.00^{d}±0.00	2.22^{a}±0.04
Coliform (log_{10} cfu/g FM)	3.11^{a}±0.08	2.00^{b}±0.16	2.00^{b}±0.00	2.00^{b}±0.10
Chemical composition				
Dry matter (g/kg FM)	660.84±2.06	660.13±0.55	653.76±0.64	658.63±2.52
Crude protein (g/kg DM)	110.28^{c}±2.03	118.29^{b}±2.38	113.52^{bc}±0.62	125.20^{a}±0.37

†Control: without microbes, T1: *L.plantarum* KACC11451, T2: *L.brevis* RNAL14, T3: *L.mucosae* 521129, FM: Fresh matter, DM: Dry matter.
^{a,b,c,d}Means with different superscripts within the row column differ significantly ($p < 0.05$).

Table 4. Total gas production, pH, and ammonia-nitrogen production of *in vitro* rumen fermentation using fermented total mixed ration (FTMR)

Time(h)	Treatments			
	Control	T1	T2	T3
		Total gas production (mL)		
3	20.67±0.93	20.03±0.75	18.00±1.04	19.67±1.42
6	30.17^{b}±0.83	32.83^{ab}±2.20	29.73^{b}±0.93	36.00^{a}±1.32
12	48.67±4.26	47.33±6.36	51.50±2.02	50.67±2.33
24	69.67±2.85	66.33±5.81	62.67±2.33	63.67±1.20
48	85.67±0.33	86.33±0.66	87.00±1.53	86.67±1.20
		pH		
0	6.70^{a}±0.05	6.47^{b}±0.05	6.48^{b}±0.03	6.53^{b}±0.02
3	5.98±0.04	6.01±0.01	6.01±0.01	6.04±0.02
6	5.59±0.02	5.56±0.04	5.60±0.02	5.51±0.02
12	5.26±0.01	5.25±0.01	5.23±0.01	5.26±0.00
24	5.20±0.03	5.19±0.03	5.18±0.02	5.23±0.00
48	5.13±0.02	5.08±0.01	5.08±0.03	5.08±0.02
		Ammonia-nitrogen concentration (mM)		
0	6.39^{c}±0.25	7.44^{ab}±0.08	7.96^{a}±0.20	6.80^{bc}±0.23
3	6.86±0.00	8.76±0.44	9.59±0.65	8.46±1.37
6	7.73±0.23	9.01±0.00	9.22±1.12	8.89±0.88
12	8.95±0.13	8.72±0.08	9.17±0.00	9.33±0.03
24	12.62±0.50	13.11±1.07	12.73±0.92	13.11±1.30
48	13.11±0.63	13.36±1.43	15.00±0.03	15.49±1.83

^{a,b,c}Means with different superscripts in the same column differ significantly ($p < 0.05$).
Control: without microbes, T1: *L.plantarum* KACC11451, T2: *L.brevis* RNAL14, T3: *L.mucosae* 521129

NH_3-N at an early stage of incubation. The pH did not differ until 48 h, however, the pH of *Lactobacillus* treated TMR began to low after 48 h incubation compared to that of the control. Total gas production and NH_3-N also did not differ among treatments at 48 h. Similar to pH, NH_3-N in the *Lactobacillus* treated TMRs became higher than that in the control after 48 h.

Table 5 shows the volatile fatty acid (VFA) production of *in vitro* rumen fermentation. The total VFA concentration, for

Table 5. Volatile fatty acid production of in vitro rumen fermentation using fermented total mixed ration (FTMR)

Time(h)	Treatments			
	Control	T1	T2	T3
		Acetate (mM)		
0	23.37^{a}±0.40	21.70^{b}±0.08	24.08^{a}±0.38	22.26^{b}±0.13
3	26.49±0.41	27.26±0.00	27.36±0.18	26.51±0.41
6	29.32^{b}±0.28	30.03^{ab}±0.27	30.66^{a}±0.26	31.00^{a}±0.54
12	33.06±0.47	33.04±0.80	35.06±0.39	33.86±0.41
24	35.38^{d}±0.04	35.72^{c}±0.04	37.36^{a}±0.18	36.48^{b}±0.08
48	34.62±0.50	34.99±0.37	36.57±0.56	36.16±0.62
		Propionate (mM)		
0	5.19±0.12	5.11±0.03	5.15±0.05	5.21±0.02
3	8.36±0.14	8.28±0.18	8.02±0.01	8.06±0.02
6	10.25^{c}±0.08	11.46^{a}±0.14	10.23^{c}±0.04	11.06^{b}±0.14
12	12.84±0.24	13.20±0.51	12.88±0.25	13.59±0.53
24	14.73±0.21	14.91±0.10	14.77±0.39	15.33±0.16
48	14.80±0.12	15.03±0.29	14.55±0.33	14.80±0.32
		Butyrate (mM)		
0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
3	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
6	4.58^{b}±0.12	5.16^{a}±0.15	4.66^{b}±0.15	5.20^{a}±0.11
12	7.68±0.35	7.83±0.74	7.43±0.28	7.65±0.31
24	11.35^{b}±0.46	12.57^{a}±0.27	10.68 ^{bc}±0.01	10.26^{c}±0.02
48	14.89±0.22	16.29±0.39	15.41±0.43	14.95±0.34
		A:P		
0	4.51^{a}±0.70	4.25^{b}±0.04	4.67^{a}±0.11	4.27^{b}±0.04
3	3.17^{b}±0.01	3.29^{ab}±0.07	3.41^{a}±0.02	3.29^{ab}±0.05
6	2.86^{b}±0.01	2.65^{c}±0.01	3.00^{a}±0.02	2.80^{b}±0.03
12	2.58^{ab}±0.01	2.51^{b}±0.04	2.72^{a}±0.02	2.50^{b}±0.09
24	2.40±0.04	2.40±0.02	2.53±0.08	2.38±0.02
48	2.34±0.03	2.33±0.07	2.52±0.09	2.45±0.09
		Total VFA (mM)		
0	28.56^{a}±0.49	26.81^{b}±0.05	29.23^{a}±0.36	27.47^{b}±0.12
3	34.85±0.55	35.54±0.18	35.38±0.18	34.58±0.41
6	44.14^{b}±0.45	46.95^{a}±0.55	45.55^{ab}±0.42	47.26^{a}±0.76
12	53.58±1.06	54.07±2.05	55.37±0.92	55.10±1.12
24	61.46^{c}±0.43	63.20^{a}±0.20	62.81^{ab}±0.24	62.07^{bc}±0.22
48	64.31^{b}±0.38	66.32^{a}±0.47	66.52^{a}±0.09	65.90^{a}±0.71

^{a,b,c} Means with different superscripts in the same column differ significantly ($p < 0.05$). Control: without microbes, T2: L.brevis RNAL14, T3: L.mucosae 521129

all treatments, increased with increasing incubation time. Acetate concentration was highest (p<0.05) in the control (23.37mM) and T2 (24.08mM) at 0 h incubation. In addition, acetate concentration was higher (p<0.05) in the control than the treatment group after 6 and 24 h of incubation. Propionate concentration was highest (p<0.05) in the T1 (11.46 mM) at 6 h incubation. Also, butyrate concentration was highest (p<0.05) in T1 at 6 h and 12 h incubation. AP ratio was highest (p<0.05) in the control and T2 at 0 h incubation. In addition, the AP ratio was highest (p<0.05) in T2 at 3, 6, and 12 h incubation. At 0 h, the total VFA concentration was highest (p<0.05) in the control (28.56 mM) and T2 (29.23 mM). Additionally, total VFA concentration was higher (p<0.05) in the control than the treatment group after 6, 12, 24, and 48 h of incubation. Overall, we found no differences in acetate and butyrate concentration after 48 h of incubation. However, a tendency towards higher acetate and butyrate concentration in treatment groups compared to the control were observed after 48 h. Nevertheless, at 48 h of incubation, total VFA concentration was higher (p<0.05) in the treatment groups than the control. Especially, total VFA concentration differed significantly among the treatments with the highest value observed in the T2 treatment.

Ⅳ. DISCUSSION

Three LAB (*L. plantarum*, *L. brevis*, and *L. mucosae*) were used as TMR fermention starter in this study. *L. plantarum* is often used to secure desirable lactic acid fermentation (Weinberg and Muck, 1996) while *L. mucosae* increased propionate and total VFA concentrations in the fermentation of brewers grains and the *in vitro* rumen fermentation (Mamuad et al., 2017, Soriano et al., 2014). Inoculation of LAB spp. in TMR could lower pH and increase the concentrations of lactic acid, acetic acid, and total organic acid compared to non inoculated control TMR (p<0.05). In addition to the increased acidity and VFA concentration, the numbers of aerobic bacteria and LAB were higher, but *E. coli* counts were lower compared to the control group (p<0.05). Notably, the *L. plantarum* treatment, which had the lowest pH, highest lactic acid content and the highest numbers of aerobic bacteria and LAB, also showed the highest numbers of fungi. Fungi are sensitive to products of fermentation such as lactic acid and acetic acid (Bonestroo et al., 1993). Hetero-fermentative LAB produces acetic acid and propionic acid, and lactic acid causes the main decrease in pH (Eklund, 1989). Propionic acid, in particular, shows characteristic inhibition of fungal growth (Woolford, 1984). Furthermore, this is consistent with a report by Voulgari et al, (2010), in which an anti-fungal test using ATCC14917, which is the same strain as the *L. plantarum* KACC11454 used in the T1 treatment. In contrast, the *L. brevis* treatment had the highest concentration of total organic acid and fungus was not detected, while the *L. mucosae* treatment had high acetic acid contents and also showed the highest crude protein content.

In fermentation by ruminal microorganisms, gas production and pH value is an important index to measure fermentative characteristics (Lee et al., 2013). In 6 hours of fermentation, the *L. mucosae* treatment had the highest of total gas production (p<0.05), had the lowest pH, but this was not statistically significant (p>0.05), and showed the highest concentration of total VFA (p<0.05). Although there was no statistically significant difference in total gas produced and pH in the LAB-treated group after 48 hours of fermentation, LAB fermentation of TMR feed is considered to increase ruminal fermentation, since the total volatile fatty acids produced were high (p<0.05).

The fermented TMR feed added with LAB briefly showed a lower pH than the control group at 0 hours, due to the abundant production of lactic acid and acetic acid, but the pH became similar level to the control by 3 hours of fermentation. The concentration of ammonia nitrogen was higher in the LAB-treated group at 0 h fermentation (p<0.05) and it was maintained with higher trend level throughout the whole fermentation period, even though this was not statistically significant (p>0.05). Fermented TMR feed with added LAB contains a high level of crude proteins compared to the control, which suggests that more active proteolysis by ruminal microorganisms could have occurred. Also, like a previous report by Stiles et al, (1970), the concentration of ammonium nitrogen was confirmed to be at an appropriate level. Ultimately, fermented TMR feed with added LAB increased the production of total volatile fatty acids compared to fermented TMR with only water added. There was no great difference between strains of LAB.

Ⅴ. CONCLUSION

The use of LAB, especially *L. brevis* as an inoculant for fermentation of high moisture TMR could inhibit fungi growth and promote lactic fermentation, and enhance digestion in the rumen.

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