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Effects of Synbiotics Containing Anaerobic Microbes and Prebiotics on *In vitro* Fermentation Characteristics and *In situ* Disappearance Rate of Fermented-TMR

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ABSTRACT : This study was carried out to estimate effects of synbiotics containing anaerobic microorganisms and prebiotics on *in vitro* fermentation characteristics and *in situ* disappearance rate of fermented total mixed ration (F-TMR). For the *in vitro* trial, ninety vinyl bags were prepared to analyze temperature, pH, ammonia concentration, microbial growth rate and short chain fatty acid concentration. For the *in situ* trial, one hundred twenty nylon bags were prepared to analyze dry matter (DM), acid detergent fiber (ADF) and neutral detergent fiber (NDF) disappearance rate. Treatments consisted of a basal diet (US) with prebiotics and probiotics from anaerobic mold (MS), bacteria (BS), yeast (YS) or compound (CS). It was found that temperatures at 14 and 21 days were significantly higher ($p<0.05$) in the YS and CS than in the others. The pH at 21 days was lower in the CS than in the US. The synbiotic treatments had significantly increased ($p<0.05$) ammonia concentration at 21 days. The DM disappearance at 72 h was significantly higher ($p<0.05$) in the MS and CS than in the others. ADF and NDF disappearance rate tended to increase at a rate similar to the DM disappearance rate. Therefore, this study suggests that synbiotics (probiotics with prebiotics) may partially help the quality of fermentation and digestibility of TMR (MS and CS) as fiber disappearance. (**Key Words :** Anaerobic Microbes, Fermented-TMR, Probiotics, Synbiotics)

INTRODUCTION

Considerable food, beverages and agricultural by-products are burned and buried into landfills, which leads to world wide economic and environmental concerns due to such wasteful disposal (Cao et al., 2010). The utilization of these by-products following inoculation with microorganisms to improve their digestibility, nutritive value and palatability may also decrease environmental problems through a reduction of agricultural waste. Filya (2003) reported that some silages are inoculated with certain microbes to speed up the fermentation process or prevent spoilage and improve the quality of silage.

Total mixed ration (TMR) was developed in North America during 1950's to 1960's (Mccullough, 1991) and produced by a method that combines all forages, by-products, grains, protein supplements, minerals and vitamins that have been mixed together to make a balanced ration in which the weight of each ingredient is known. Since all the forages, by-products, grains, protein and mineral-vitamin supplements are blended together cows are less able to selectively consume individual ingredients. TMR also helps maintain rumen pH and acetate to propionate ratio because it provides a more balanced ration with a uniform rate of roughage and concentration and increased DM intake (Li et al., 2003). Therefore, each bite of a TMR ration will include the same proportion of forages and concentrates (Ha et al., 2005).

The fermentation process of silage can be classified into five stages. In the first stage, carbon dioxide is discharged by respiration of the plant ensiled in the silage; in the second stage coli-type bacteria and other fungi produce acetic acid; in the third stage, LAB produce lactic acid and the lactic fermentation begins; in the fourth stage, lactic acid content reaches a peak and pH maintains at 4.2 or less. The third stage is completed after silage is ensiled for 3 days, and the fourth stage finishes in 17 to 21 days. In the fifth stage, if water-soluble carbohydrates are insufficient

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and pH does not fall sufficiently, butyric acid bacteria produce butyric acid (Barnett, 1954). Fermented-TMR (F-TMR) has been manufactured by inoculating some microbes to improve the fermentation quality. There are several potential advantages of feeding a F-TMR to cows which are: improving cow health, DM intake, feed efficiency, reproductive performance and milk production, reducing feed costs, labor requirements, metabolic diseases and widening the range of choices through using by-products (Ha et al., 2005). Furthermore, the study of F-TMR as a method to minimize environmental impacts such as reduction of methane production has gradually increased (Ha et al., 2005).

In recent years, probiotics, prebiotics and synbiotics were used as an additional inoculation to diets. Kelly (1998) defined probiotics as a live microbial feed supplement, which beneficially affects the host animal through improved intestinal balance. The main effects of probiotics are the advanced resistance to pathogenic bacteria colonization and improved host mucosa immunity, which results in a decreased pathogen load and enhanced health status of animals (Choct, 2009) resulting in a reduced risk of food-borne pathogens. Prebiotics are ingredients such as starch, fiber and oligosaccharides that beneficially affect the host by selective stimulation of the growth and activity of one or a limited number of bacterial species already resident in the colon (Gibson and Roberfroid, 1995).

Synbiotics are defined as a mixture of probiotics and prebiotics. Generally, homo-fermentative lactic acid bacteria such as *Enterococcus faecium*, *Lactobacillus plantarum* and *Pediococcus* spp. have been used to inoculate silages. Hetero-fermentative *Lactobacillus* such as *Lactobacillus buchneri* has been studied recently for silages (Weinberg et al., 1999; Ranjit and Kung, 2000). The effects of inoculating silage with *Lactobacillus* spp. can improve silage fermentation (Stokes, 1992) due to a decrease in pH, ammonia and acetate concentrations, and increase in lactate concentration acetate. It is believed that the inoculated populations of *Lactobacillus* spp. become dominant in silage, thereby leading to increased lactic acid concentration, decreased pH and gas, and protein decomposition (Inglis et al., 1999). Livestock also are fed *Lactobacillus* spp. to improve intestinal microbial balance due to elimination or reduction of undesirable microorganisms (Zhao et al., 1998).

Supplemental yeast and yeast culture added to the diet of lactating cows changes ruminal fermentation characteristics (Martin and Nisbet, 1990) and improves ruminal digestibilities of crude protein (CP) and crude fiber (CF) (Gomez-Alarcon et al., 1990; Williams et al., 1991). *Saccharomyces cerevisiae* contains low fat, high protein and vitamin levels (Kim and Park, 1997) and has the effect of removing residual oxygen due to a high affinity for oxygen (Rose, 1980) as well as decreasing pH and increasing CP (Ok et al., 2006). Moreover, supplemental fungi and fungal extracts have been utilized to improve the nutritional values of the diet due to enzymes produced by fungi (Gomez-Alarcon et al., 1990; Martin and Nisbet, 1990) such as amylase, xylanase and protease (Pettersson et al., 1989) that eases the digestion and aids in the absorption of nutrients (Graham et al., 1988). *Aspergillus oryzae* produces enzymes that can break down polysaccharides such as cellulases, hemicellulases and exterases (Roper and Fennel, 1965) which improves dry matter digestibility (Gomez-Alarcon et al., 1990) and increases milk or fat-corrected milk production (Kellems et al., 1990).

Although inoculants have been used for several decades, there are still unanswered questions about the interaction of inoculant *Lactobacillus* with other microbes and how this interaction drives fermentation and affects utilization of the silage by animals (Weinberg and Muck, 1996). TMR has an affect on the rumen fermentation characteristics by influencing the microbial population (Li et al., 2003). However, the fermentation characteristics and disappearance rates of NDF and ADF have not been studied when F-TMR was used *in vitro* or *in situ* trials. Therefore, ***the*** objective of this study was to determine the effects of supplemental probiotics containing anaerobic microbes (mold, bacteria and yeast) mixed with prebiotics, such as mannan oligosaccharide, sodium acetate, lactose and ammonium citrate on *in vitro* fermentation characteristics and *in situ* disappearance of F-TMR.

MATERIALS AND METHODS

Experimental diets

The TMR was provided by Korea Federation of Cooperatives (Sancheong, Korea). The TMR ingredients contained 27.80% brewer's grain, 25.00% corn flakes and 11.00% dehulled soybean meal, 7.60% corn craking, 7.40% rice straw, 4.00% molasses, 3.00% perennial ryegrass, 2.60% barley craking, 2.20% corn gluten and others (Table 1). The chemical composition of the experimental diet was 9.12% CP, 2.74% crude fat, 6.26% ash and 31.69% nitrogen free extract (NFE). The cell wall constituents of the experimental diet contained 33.26% neutral detergent fiber (NDF), 15.32% acid detergent fiber (ADF), 17.94% hemicellulose and 10.98% cellulose, respectively (Table 2).

Design of *in vitro* trials

Ninety TMR (5 treatments×6 incubations×3 replications) were processed in a laboratory scale mini-silo to investigate the effects of the supplemental probiotics with prebiotics on *in vitro* fermentation characteristics and *in situ* disappearance of dry matter (DM) and fiber of F-TMR. Probiotics were made from anaerobic microbes (bacteria, yeast and mold) and prebiotics as mannan oligosaccharide,

Table 1. Ingredient composition of basal diets for the current experiment

Items	Concentration
Ingredients, as-fed basis (%)	
Brewer's grains	27.80
Corn flake	25.00
Dehulled soybean meal	11.00
Corn craking	7.60
Rice straw	7.40
Molasses	4.00
Perennial ryegrass	3.00
Barley craking	2.60
Corn gluten	2.20
Full fat cotton	1.60
Cereal bran	1.60
Soy sauce cake	1.40
Perilla meal	1.40
Cotton pellet	1.00
Beet pulp	1.00
Limestone	0.80
Salt	0.40
Butter milk	0.20

sodium acetate, lactose and ammonium citrate.

The 5 dietary treatments were: i) US, basal diet, ii) MS, basal diet+probiotics from anaerobic mold+prebiotics, iii) BS, basal diet+probiotics from anaerobic bacteria+ prebiotics, iv) basal diet+probiotics from anaerobic yeast+ probiotics from anaerobic mold+prebiotics v) CS, basal diet +probiotics from anaerobic mold, bacteria and yeast+ prebiotics. *Lactobacillus* spp., *Saccharomyces cerevisiae* and *Aspergillus oryzae* were used as probiotics in BS, MS and YS diets and were cultured in Difco^{TM} *Lactobacilli* De Man, Rogosa and Sharpe (MRS) Broth at 37°C (incubation for 24 h), Difco^{TM} Potato Dextrose Broth at 30°C (incubation for 36 h) and Difco^{TM} YM Broth at 30°C (incubation for 24 h), respectively. They were cultured to saturation density. The medium for probiotics from anaerobic bacteria was 10 g proteose pepton No. 3, 10 g beef extract, 5 g yeast extract, 20 g dextrose, 1 g polysorbate 80, 2 g ammonium citrate, 5 g sodium acetate, 0.1 g magnesium sulfate, 0.05 g manganese sulfate and 2 g dipotassium phosphate per liter. The media for Probiotics from anaerobic mold was 20 g dextrose and 4 g potato starch per liter. The medium for probiotics from anaerobic yeast was 3 g yeast extract, 3 g malt extract, 10 g dextrose and 5 g peptone per liter. All cultures were carried out under anaerobic conditions. Anaerobic culture system was prepared in accordance with methods of Holdman et al. (1977).

In this study, 100 ml of the selected microbes (control

Table 2. Chemical composition and energy content of basal diet

Items	Concentration
Proximate analysis, as-fed basis (%)	
Moisture	40.65
Crude protein	9.12
Crude fat	2.78
Crude fiber	12.28
Ash	6.26
Nitrogen free extracts	28.91
Cell wall constituents[1] (DM basis, %)	
Neutral detergent fiber (NDF)	33.26
Acid detergent fiber (ADF)	15.32
Hemicellulose	17.94
Cellulose	10.98
Potassium permanganate lignin	2.97
Lignin/NDF[2]	8.93
Lignin/ADF[3]	19.39

[1] Analytical values.
[2] Lignification index based on neutral detergent fiber.
[3] Lignification index based on acid detergent fiber.

was distilled water) were added to vinyl bags (43 cm×58 cm) that contain 3 kg TMR and sealed perfectly using Vacuum Packaging Machine (AZ-450, Intrise, Korea), then fermented at room temperature (RT). Temperature, pH, ammonia concentration, microbial growth rate and concentration of short chain fatty acids were measured on the fermented samples.

At each fermentation period, 1.5 ml aliquot of the F-TMR was frozen prior to determining the concentration of short chain fatty acid and lactic acid. Three samples were taken into 1.5 ml tubes from each treatment after thawing previously frozen samples. Then samples were centrifuged at 3,000 *g* for 3 min at 4°C to remove feed particles before filtering them through 0.20 μm syringe filter (37070 Goettingen, Sartorius Biotech GmbH, Germany). The filterates were analyzed to measure the concentrations of short chain fatty acid and lactic acid with the use of high performance liquid chromatography (HPLC) in a Varian column (USA) at 35°C, isocratic elution with 0.0085 N sulfuric acid, and UV/Visible detection (Agilent, Germany) at 210 nm and 220 nm wave lengths. A mixture of acetic, lactic, formic, butyric and propionic acids was included as a standard in all analyses.

Analysis of *in vitro* trials

TMR samples from *in vitro* trials were obtained from each mini-silo and dried at 65°C for 3 days, ground and screened through a 1 mm screen in a Willey mill to analyze ingredient composition, chemical composition and energy contents by AOAC (1995). The NFE concentration was determined by an organic matter formula (AOAC, 1995).

Concentrations of NDF and ADF were determined by the method of Van Soest et al. (1991).

The temperature of F-TMR was determined by inserting a thermometer into the central part of F-TMR at certain time (12.00 h) after opening the silage bags at fermentation periods (1, 3, 5, 7, 14 and 21 d).

The pH value was immediately measured using a pH meter (MP-230, Mettler toledo, Switzerland) on the filtrate that was squeezed through 4 layers of cheesecloth after blending 50 g of F-TMR in 450 ml of distilled water using homogenizer (SS-20D, Young Hana TECH, Korea).

Ammonia concentration was analyzed by the method of Chaney and Marbach (1962). An aliquot of 0.06 ml mercury chloride was added to 6.2 ml aliquot of sample to stop microbial activity and centrifuged at 3.000×g for 10 min. The 0.02 ml supernatant, distilled water and ammonia standard solution (5, 10 and 20 mg NH_3-N/100 ml) were inserted into each 20 ml tube. Then the supernatant aliquotes and standard solutions were mixed with aphenol color reagent and alkali-hypochlorite reagent. Ammonia concentration was determined using spectrophotometer (Model 680, BIO-RAD, Japan) at 630 nm wave length.

To determine the microbial growth rates, F-TMR samples taken from each fermentation period were centrifuged at 3,000 g for 3 min to remove feed particles and then the supernatants were re-centrifuged at 14,000 g for 3 min to settle the pellets down. After that, sodium phosphate buffer (pH 6.5) was added to these precipitates and stirred. Growth rates of all microbes were estimated as optical density (OD) values using spectrophotometer (Model 680, BIO-RAD, Japan) at 550 nm. This process was repeated three times and means were determined.

Design and analysis of *in situ* trial

Samples were collected from F-TMR fermented for 21 d. One hundred twenty nylon bags (5 treatments×8 incubations×2 replications) of 3 g for *in situ* experiments were collected at 1, 3, 6, 9, 18, 24, 48 and 72 h to estimate the disappearance rates of DM and fiber as NDF and ADF from the bag in the rumen.

Two ruminally and duodenally cannulated dairy cows (550 kg of average body weight) were used to determine *in situ* DM disappearance under confined feeding and housing conditions. Diet was formulated to meet or exceed National Research Council (NRC, 2001) recommendations for lactating dairy cows and water was offered at all times. One hundred twenty F-TMR nylon bags including 5 treatments ×8 incubations with duplicates were processed for the *in situ* trial. Nylon bags (45 μm pore size, NYTAL 25T, Swiss screen P/L Co. Ltd, Swiss) were filled with 3 g samples of dried F-TMR that had been ground previously and screened through a 1 mm screen in a Wiley mill. All bags were soaked in tepid water for 10 min to remove water soluble components and reduce lag time associated with wetting. Subsequently, all bags were inserted into the ventral rumen of two dairy cows simultaneously just before feeding and incubated for 1, 3, 6, 9, 18, 24, 48 and 72 h. Upon removal, each sample was washed for 3 min until the rinse water remained clear, frozen for later analysis and then dried to a constant weight at 60°C for 48 h (Van Keuren and Heineman, 1962). *In situ* DM disappearance was calculated with initial DM concentration and DM concentration of finished fermentation.

The DM was partitioned into three fractions based on relative susceptibility to ruminal DM disappearance and the degradation parameters of DM for the experimental F-TMR were calculated as the following equation (Orskov and McDonald, 1979);

$$P = a+b(1-e^{-ct}),$$

Where:
P = DM disappearance at time t,
a = percentage of rapidly soluble fraction, %
b = insoluble potentially degradable fraction, %
c = constant rate of disappearance for b fraction, per hours
t = fermentation time in the rumen, /h

Effective ruminal degradability (ERD) of DM was calculated at 4.0%/h (National Research Council; NRC 1984) of passage rate for F-TMR from the equation (Orskov and McDonald, 1979);

$$ERD = a+b\times(c/(c+r)),$$

Where:
ERD = effective ruminal degradability
a, b, c = same as in DM disappearance
r = ruminal passage rate (4.0%/h)

The samples obtained from *in situ* trial were dried at 60°C for at least 48 hours before measurement of NDF and ADF disappearances. Residual matter was analyzed for NDF and ADF contents. Percentage of NDF and ADF of the original sample was determined and used to calculate percentage of indigestible NDF and ADF. Disappearance of NDF and ADF in nylon bags was determined by the methods described by Van Soest (1991). Ankom system (Ankom 220 fiber analyzer, Ankom, USA) was used for analysis of samples and ADF and NDF were calculated based on the equation;

NDF (ADF) disappearance (%)
= NDF (ADF) contents before fermentation
-NDF (ADF) contents after fermentation

NDF (ADF) contents before fermentation

Statistical analyses

The effect of *in vitro* dietary treatment was examined in a one-way ANOVA model that included treatment and incubation time. The effect of *in situ* dietary treatment was examined in two-way ANOVA model that included treatment, incubation time and animal. Data were analyzed as repeated measures using the General Linear Model (GLM) procedure of SAS (1999). Duncan's Multiple Range Test method at $p<0.05$ (Duncan, 1955) was used to test the significance of differences among means.

RESULTS AND DISCUSSION

In vitro trial

The effects of supplemental synbiotics and fermentation periods on temperature and pH of F-TMR during the fermentation periods are shown in Table 3. Fermentation occurred relatively steadily for all silages as shown by increase in silage temperature over the 21 d of ensiling. The temperature increased by 3°C over all fermentation periods. The temperature of YS and CS was significantly higher ($p<0.05$) than US at 14 d. The temperature of MS and CS was significantly higher ($p<0.05$) than that of US at 7 d but MS was not different from US as well as BS at 14 and 21 d. The pH value constantly declined as the F-TMR fermented, which ranged from 4.42 to 4.56 at the last fermentation period. The pH value was significantly lower ($p<0.05$) in YS than in US at 1 d, while MS, BS, YS and CS, which were inoculated with synbiotics tended to be similar to US at 21 d. The pH values of CS at 3 d and 7 d were significantly ($p<0.05$) lower than other treatments as well as BS, respectively. However, there was a significant difference among treatments as the fermentation process proceeds.

Silage that contains a high level of moisture and slightly higher level of DM with a pH value under 4.0 and over 4.0, respectively, is considered of good quality (McDonald, 1981). Thus, pH ranges of this experiment indicate good quality of silage.

Ammonia concentration was not modified by the inoculation of synbiotics (Table 4). A non-significant increase up to 14 d was observed and then increased with an extended fermentation period, but no interaction was found with the ammonia concentration of F-TMR. Ruminal mold goes through a series of changes and developments, starting with the release zoospores after 15 min to 1 h of feeding and then continues through several stages such as germination, development of rhizoid, differentiation and etc. This life cycle lasts for 24 to 30 h in the rumen but for 3 d in *in vitro* trials (Ha et al., 2005). Therefore, the higher ammonia concentration in MS compared with other treatment at 3 d, could be a result of proliferation of mold. Generally, an ammonia concentration of less than 10 mg/dl is an indication of good quality silage (Martinsson, 1991). In this study, all of the treatments showed ammonia concentrations that ranged from 2.20 to 5.07 mg/dl, which

Table 3. Effects of supplemental probiotics from anaerobic microflora mixed with prebiotics on the internal temperature and pH values in F-TMR[1]

Fermentation period (d)	Treatment[2]					Mean[3]	SEM[4]
	US	MS	BS	YS	CS		
Temperature (°C)							
1	23.13[b]	23.43[ab]	23.20[ab]	23.50[a]	23.20[ab]	23.29	0.16
3	24.20	24.20	24.30	24.37	24.23	24.30	0.19
5	22.93[c]	23.40[b]	23.37[bc]	23.50[b]	24.07[a]	23.45	0.24
7	24.27[bc]	24.37[a]	24.23[c]	24.33[ab]	24.40[a]	24.32	0.05
14	24.80[b]	25.10[ab]	25.00[ab]	25.30[a]	25.13[a]	25.07	0.17
21	26.20[b]	26.45[b]	26.47[b]	27.27[a]	26.30[b]	26.57	0.29
pH							
1	4.98[a]	4.81[ab]	4.94[a]	4.71[b]	4.79[ab]	4.85	0.11
3	4.66[b]	4.69[ab]	4.77[a]	4.71[ab]	4.54[c]	4.67	0.05
5	4.61	4.60	4.63	4.49	4.49	4.56	0.08
7	4.51[ab]	4.48[ab]	4.57[a]	4.51[ab]	4.43[b]	4.50	0.06
14	4.46	4.41	4.40	4.38	4.40	4.41	0.04
21	4.56	4.52	4.51	4.52	4.42	4.50	0.07

[1] *In vitro* trial, mean of 3 total mixed ration (TMR) vinyl bags individually analysed in each fermentation period.
[2] The total mixed ration was supplemented with probiotics and prebiotics. US = Un-supplemental probiotics and prebiotics; MS = Probiotics from anaerobic mold with prebiotics; BS = Probiotics from anaerobic bacteria with prebiotics; YS = Probiotics from anaerobic yeast with prebiotics; and CS = Combined probiotics anaerobic bacteria, yeast and mold with prebiotics. Prebiotics used mannan oligosaccharide, sodium acetate, lactose and ammonium citrate.
[3] Mean of all treatment values in the same row (fermented periods). [4] Standard error of the mean.
[a,b,c] Values in the same row with different superscripts differ at $p<0.05$.

Table 4. Effects of supplemental probiotics from anaerobic microflora mixed with prebiotics on the ammonia concentration and microbial growth rate in F-TMR[1]

Fermentation period (d)	Treatment[2]					Mean[3]	SEM[4]
	US	MS	BS	YS	CS		
Ammonia (mg/dl)							
1	3.61	2.20	2.84	3.82	2.44	2.98	0.89
3	2.80[b]	3.65[a]	2.87[b]	2.42[b]	2.87[b]	2.92	0.33
5	2.68[b]	2.76[b]	3.78[a]	3.55[a]	2.60[b]	3.07	0.32
7	3.48	2.36	3.63	2.94	3.05	3.09	0.87
14	3.41	2.98	2.98	2.49	3.41	3.06	0.78
21	3.89[cd]	5.07[a]	4.31[bc]	4.71[ab]	3.30[d]	4.25	0.37
Microbial growth rate (Optical density)[5]							
1	0.14[b]	0.15[b]	0.21[ab]	0.17[ab]	0.23[a]	0.18	0.04
3	0.15	0.26	0.28	0.25	0.26	0.24	0.07
5	0.29	0.29	0.24	0.31	0.40	0.31	0.09
7	0.46	0.52	0.43	0.49	0.56	0.49	0.49
14	0.45	0.48	0.53	0.48	0.57	0.50	0.51
21	0.32	0.32	0.39	0.35	0.39	0.35	0.35

[1] *In vitro* trial, mean of 3 total mixed ration (TMR) vinyl bags individually analysed in each fermention period.

[2] The total mixed ration was supplemented with probiotics and prebiotics. US = Un-supplemental probiotics and prebiotics; MS = Probiotics from anaerobic mold with prebiotics; BS = Probiotics from anaerobic bacteria with prebiotics; YS = Probiotics from anaerobic yeast with prebiotics; and CS = Conbined probiotics anaerobic bacteria, yeast and mold with prebiotics. Prebiotics used mannan oligosaccharide, sodium acetate, lactose and ammonium citrate.

[3] Mean of all treatment values in the same row (fermented periods). [4] Standard error of the mean.

[5] Microbial growth rates were estimated as optical density value by using spectrophotometer at 550 nm.

[a,b,c] Values in the same row with different superscripts differ at p<0.05.

could be an indication of high quality of F-TMR.

Whiter and Kung (2001) reported that inoculation with anaerobic microorganisms has a beneficial effect due to rapid decline in pH value that results in less proteolysis and therefore lower concentrations of ammonia. Also, microbes inoculated into TMR are more likely to be cellulolytic microorganisms rather than proteolytic microorganisms. Ammonia concentration of F-TMR in this study was affected by inoculation of synbiotics and fermentation period, and all treatments showed significantly low concentrations of ammonia, which indicate high quality silage.

The changes of microbial growth rates are depicted in Table 4. The microbial growth rates were not significantly different among all of the treatments except for 1 d. A non-significant difference of microbial growth rates up to 3 d was observed among the treatments. However, the growth rate suddenly increased before 7 d and declined again at 21 d. To conclude, there is no significant difference of microbial growth rates due to synbiotics supplementation and *in vitro* fermentation period.

Concentration of short chain fatty acid based on fermentation period is shown in Table 5. Concentration of acetic acid reached a peak at 7 d and 14 d, and then declined at 21 d. Acetic acid of MS, YS and CS at 1 d was significantly higher ($p<0.05$) than US. No significant difference in concentration of acetic acid was observed at the early fermentation periods. However, lactic acid of MS at 14 d and YS at 21 was significantly higher ($p<0.05$) than CS and US, respectively. No interaction was found for the concentration of lactic acid and inoculation of synbiotics and fermentation period. In a previous study, short chain fatty acid contents of fermented alfalfa inoculated with bacterial probiotics for a certain period of time showed increased concentration of acetic acid for both inoculated and un-inoculated treatment (Kung et al., 2003).

Ranjit and Kung (2000) reported that inoculation with *Lactobacillus buchneri* was ineffective to increase the acetic acid concentration in corn silage. In most silages, the concentration of lactic acid is usually greater than the concentration of acetic acid with typical ratios of approximately 3 to 1 (Ward, 2000). In this study, the ratio of acetic acid to lactic acid was between 2 to 1 and 3 to 1 at all fermentation periods and was similar to the study of Driehuis et al. (2001) and Ward (2000).

In situ trial

The DM disappearance obtained from each nylon bag is shown in Table 6. There is no significant difference for 18 h of incubation time among all treatments. The DM disappearance at 72 h was significantly higher ($p<0.05$) in the MS and CS than in other treatments. The BS, YS and CS had a similar *in situ* soluble DM fraction, which was higher than that of US. However, the slowly degradable DM

Table 5. Effects of supplemental probiotics from anaerobic microflora mixed with prebiotics on the concentration of short chain fatty acid in F-TMR[1]

Fermentation period (d)	Treatment[2]					Mean[3]	SEM[4]
	US	MS	BS	YS	CS		
Acetic acid (mM)							
1	1.09[c]	1.51[b]	0.93[c]	2.29[a]	1.61[b]	1.48	0.13
3	1.30	2.36	2.55	1.48	1.77	1.89	0.70
5	2.80	2.50	2.21	2.76	2.95	2.64	0.50
7	3.00	3.23	3.50	3.14	2.70	3.11	0.50
14	2.71	3.35	3.18	3.31	2.38	2.99	0.98
21	0.77	1.01	2.63	2.68	2.22	1.86	0.79
Lactic acid (mM)							
1	0.87	0.70	1.33	0.59	0.73	0.84	0.35
3	0.38	1.16	0.79	0.40	0.46	0.64	0.29
5	0.57	0.35	0.44	0.42	0.44	0.44	0.10
7	0.63	0.50	0.61	0.51	0.50	0.55	0.11
14	0.66[ab]	0.97[a]	0.82[ab]	0.60[ab]	0.38[b]	0.69	0.17
21	0.13[b]	0.63[ab]	1.30[ab]	1.56[a]	0.42[ab]	0.81	0.48

[1] *In vitro* trial, mean of 3 total mixed ration (TMR) vinyl bags individually analysed in each fermention period.

[2] The total mixed ration was supplemented with probiotics and prebiotics. US = Un-supplemental probiotics and prebiotics; MS = Probiotics from anaerobic mold with prebiotics; BS = Probiotics from anaerobic bacteria with prebiotics; YS = Probiotics from anaerobic yeast with prebiotics; and CS = Conbined probiotics anaerobic bacteria, yeast and mold with prebiotics. Prebiotics used mannan oligosaccharide, sodium acetate, lactose and ammonium citrate.

[3] Mean of all treatment values in the same row (fermented periods). [4] Standard error of the mean.

[a,b,c] Values in the same row with different superscripts differ at $p<0.05$.

Table 6. Effects of supplemental probiotics from anaerobic microflora mixed with prebiotics on the disappearance rate of dry matter and degradation parameters in F-TMR[1]

Items	Treatment[2]					Mean[3]	SEM[4]	p	
	US	MS	BS	YS	CS			Diet	Animal
Disappearance rate of dry matter (h, %)									
1	25.98	24.76	26.47	29.56	28.13	26.98	5.68		
3	27.10	25.86	33.28	30.15	31.90	29.66	4.88		
6	33.29	31.84	35.89	35.24	32.45	33.74	5.31		
9	37.68	41.82	37.62	36.22	38.71	38.41	3.43		
18	52.22	56.22	56.77	52.26	57.27	54.95	5.66		
24	63.40[ab]	65.72[a]	57.28[b]	63.07[ab]	68.99[a]	63.69	3.39	*	
48	73.77[a]	75.20[a]	69.64[b]	69.76[b]	74.68[a]	72.61	1.86	**	
72	74.11[b]	80.69[a]	71.00[b]	73.99[b]	80.21[a]	76.00	2.52	**	
Degradation parameters of dry matter[5]									
a	16.60	20.44	24.82	24.21	22.22				
b	60.30	62.21	48.56	52.94	60.46				
c	0.0542	0.0477	0.0486	0.0428	0.0471				
Effective rumen degradability[6] (%)									
r = 0.04	51.30	54.27	51.46	51.58	54.91				

[1] Mean of 3 total mixed ration (TMR) nylon bags individually analysed in each incubation times (h).

[2] The total mixed ration was supplemented with probiotics and prebiotics. US = Un-supplemental probiotics and prebiotics; MS = Probiotics from anaerobic mold with prebiotics; BS = Probiotics from anaerobic bacteria with prebiotics; YS = Probiotics from anaerobic yeast with prebiotics; and CS = Conbined probiotics anaerobic bacteria, yeast and mold with prebiotics. Prebiotics used mannan oligosaccharide, sodium acetate, lactose and ammonium citrate.

[3] Mean of all treatment values in the same row (incubation time). [4] Standard error of the mean.

[5] Parameters were calculated by $P = a+b(1-e^{-ct})$. P = dry matter disappearance at time; a = percentage of rapidly soluble fraction; b = percentage of insoluble potentially degradable fraction; c = constant rate of disappearance for b fraction; and t = fermentation h in the rumen.

[6] Values were calculated by $ERD = a+b\times(c/(c+r))$. ERD = effective ruminal degradability; a = percentage of rapidly soluble fraction; b = percentage of insoluble potentially degradable fraction; c = constant rate of disappearance for b fraction; and r, ruminal passage rate.

[a,b] Values in the same row with different superscripts differ at * $p<0.05$ and ** $p<0.01$.

fraction between MS and CS was the highest and the rate of degradation was similar among treatments except US. Effective DM degradability was similar for MS and CS, and was higher in US, BS and YS.

Williams et al. (1990) reported that DM disappearance was increased approximately 6.8 to 16.3% when hay was incubated into rumen supplemented with a fungal culture for 12 h and then declined rather than increased, which associated molds with the initial disappearance due to rapid population growth after feeding. However, the results of this experiment showed that DM disappearance was higher after 24 h which is different from the previous report. This could be attributed to the difference of this experiment, which was not conducted on fungal culture administration to rumen but *in situ* nylon bag trial of F-TMR for certain period of time. Live yeast culture contributed to production improvement of livestock as feed additive. The literature (Williams et al., 1991) showed no improvement in *in situ* DM disappearance when *Saccharomyces cerevisae* was added to the diet, but caused small increase in fiber disappearance. Filya (2003) reported that addition of *Lactobacillus buchneri*, and *Lactobacillus plantarum* did not affect rumen degradability of silage, which was in agreement with this experiment.

The NDF and ADF disappearance calculated based on measures of *in situ* DM disappearance of F-TMR are shown in Table 7. There were no significant difference of NDF disappearance among the treatments at 1, 3 and 18 h of incubation, while the NDF disappearance at 72 h of incubation was significantly higher (p<0.05) in the MS than in the other treatments. The ADF disappearance was not different at 1, 3 and 24 h of incubation and ADF disappearance at 9 and 72 h was significantly lower (p<0.05) in the BS than in the other treatment. Also, there were no animal effect on DM, NDF and ADF disappearance in this experiment.

Most studies revealed that bacteria can be involved in digestion of feed due to metabolic diversity of bacteria (Cheng et al., 1991). However, DM and fiber disappearance of MS and CS were higher than BS in this study. It may be important to note that mold, by the nature of their mode of growth that involve hyphal extension, possess the ability to penetrate deeply into tissues normally inaccessible to bacteria, and this suggests a special role for anaerobic mold in rumen fiber digestion (Bauchop, 1981). Bauchop (1981) report that bacteria have a short life cycle and mold can slowly digest cell wall substances such as lignins, hemicelluloses, pectins and so on.

Table 7. Effects of supplemental probiotics from anaerobic microflora mixed with prebiotics on the rate of fiber disappearance in F-TMR[1]

Fermentation period (d)	Treatment[2]					Mean[3]	SEM[4]	p	
	US	MS	BS	YS	CS			Diet	Animal
Disappearance rate of neutral detergent fiber, hours, %									
1	25.23	23.35	24.33	21.06	19.54	22.70	6.06		
3	15.36	12.32	20.16	18.88	14.14	16.17	8.76		
6	20.31	20.72	13.89	14.58	22.49	18.40	4.14		
9	26.96	30.49	21.93	21.35	26.44	25.43	3.91		
18	36.36	40.51	42.49	37.10	43.04	39.90	6.59		
24	51.96[ab]	55.53[a]	43.56[b]	52.38[ab]	56.76[a]	52.04	4.79	*	
48	65.15	67.10	61.94	60.07	65.78	64.01	3.15		
72	66.84[b]	74.66[a]	59.79[c]	65.62[b]	69.95[ab]	67.37	3.02	**	
Disappearance rate of acid detergent fiber, hours, %									
1	25.90[ab]	29.40[a]	20.24[b]	19.47[b]	29.95[a]	24.99	4.19		
3	25.90	25.00	22.09	20.91	26.70	24.12	6.51		
6	25.38	30.00	19.23	20.42	28.00	24.61	4.76		
9	28.87[a]	32.34[a]	17.32[b]	25.68[a]	33.22[a]	27.48	4.02	**	
18	38.79	46.75	37.71	32.76	45.36	40.27	6.12		
24	51.22	62.10	51.62	50.51	61.20	55.33	7.25		
48	64.81[b]	67.95[ab]	58.70[c]	58.66[c]	69.70[a]	63.96	2.48	**	
72	64.01[a]	72.23[a]	52.14[b]	65.82[a]	70.11[a]	64.86	4.82	**	

[1] Mean of 3 total mixed ration (TMR) nylon bags individually analysed in each incubation times (h).

[2] The total mixed ration was supplemented with probiotics and prebiotics. US = Un-supplemental probiotics and prebiotics; MS = Probiotics from anaerobic mold with prebiotics; BS = Probiotics from anaerobic bacteria with prebiotics; YS = Probiotics from anaerobic yeast with prebiotics; and CS = Conbined probiotics anaerobic bacteria, yeast and mold with prebiotics. Prebiotics mannan oligosaccharide, sodium acetate, lactose and ammonium citrate.

[3] Mean of all treatment values in the same row (incubation time). [4] Standard error of the mean.

[a,b,c] Values in the same row with different superscripts differ at * p<0.05 and ** p<0.01.

The attachment and digestion by microbes might actively occurre in proportion to incubation time, especially lignin, cellulose and hemicelluloses, which are cell wall substances that are digested by cellulolytic microorganisms. The inoculation with *Lactobacillus buchneri*, alone or in combination with *Lactobacillus plantarum*, did not affect NDF degradability of corn and sorghum silages (Filya, 2003).

In conclusion, this study suggests that probiotics from anaerobic mold, bacteria and yeast with prebiotics may help some fermentation qualities such as increased temperature and concentration of short chain fatty acid, reduced pH, and lower level of ammonia concentration. Also, CS and MS of synbiotics can partially contribute to improvement of DM and fiber disappearance.

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