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Methane Mitigation and Microbial Diversity of Silage Diets Containing *Calliandra calothyrsus* in a Rumen *in Vitro* Fermentation System

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ABSTRACT

This study was conducted to investigate the effects of silage based diets on methane (CH_4) mitigation and microbial diversity in a rumen *in vitro* fermentation. The experiment was arranged in a completely randomized design with five treatments and three replications. The dietary treatments consisted of varying levels of silage containing 50% *Calliandra calothyrsus* as follows K; 100% concentrate + pure tannic acid of 1 mg/mL, R1; 25% silage + 75% concentrate, R2; 50% silage + 50% concentrate, R3; 75% silage + 25% concentrate, and R4; 100% silage. The fermentation variables measured were total gas, CH_4, *in vitro* organic matter digestibility (IVOMD), VFAs, pH, N-NH_3, number of protozoa, and microbial diversity analysis. Increasing level of silages reduced total gas production, CH_4 concentration, IVOMD, index of bacterial diversity, protozoal number, total methanogens and Methanobacteriales population. Diet with 25% to 50% silage decreased CH_4 concentration, total gas production and IVOMD by 11.43%, 24.92%, and 18.73%, respectively. Ammonia N and VFAs (except butyrate and valerate) were significantly reduced (P<0.01) by increasing level of silages in the ration. In conclusion, this study confirmed that 50% silage containing *C. calothyrsus* was efficient in mitigation of enteric CH_4 production by reducing total methanogens and Methanobacteriales number, but had negative effect on decreasing bacterial diversity and organic matter digestibility.

Key words: Calliandra calotyhrsus, silage, rumen fermentation, methane, microbial diversity

ABSTRAK

Penelitian ini dilakukan untuk mengevaluasi pengaruh pakan silase yang mengandung 50% *Calliandra calothyrsus* pada mitigasi metan (CH_4) dan keragaman mikroba dalam fermentasi rumen secara *in vitro*. Penelitian ini menggunakan rancangan acak lengkap dengan 5 perlakuan dan 3 ulangan. Perlakuan terdiri atas K; 100% konsentrat + asam tanat murni (1 mg/ml), R1; 25% silase + 75% konsentrat, R2; 50% silase + 50% konsentrat, R3; 75% silase + 25% konsentrat, dan R4; 100% silase. Variabel fermentasi yang diukur terdiri atas total gas, konsentrasi CH_4, kecernaan *in vitro* bahan organik (IVOMD), VFAs, pH, N-NH_3, jumlah protozoa, dan analisis keragaman mikroba rumen. Hasil penelitian menunjukkan peningkatan level silase menurunkan produksi gas total, konsentrasi CH_4, IVOMD, indeks keragaman bakteri, jumlah protozoa, populasi metanogen dan Methanobacterales. Penggunaan silase 25%-50% menurunkan CH_4, gas total dan IVOMD secara beurutan sebesar 11,43%, 24,92%, dan 18,73%. Nitrogen ammonia dan VFAs (kecuali butirat and valerat) secara nyata (P<0,01) mengalami penurunan dengan meningkatnya level silase yang digunakan. Penelitian ini menegaskan bahwa penggunaan 50% pakan silase yang mengandung *C. calothyrsus* efisien dalam mitigasi enterik CH_4 dengan menurunkan jumlah metanogen dan Methanobacteriales, tetapi masih memiliki pengaruh negatif terhadap penurunan keragaman bakteri dan kecernaan bahan organik.

Kata kunci: Calliandra calothyrsus, silase, fermentasi rumen, metan, keragaman mikroba

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INTRODUCTION

The most limiting factors in feeding cattle with forage are digestibility and nutrient quality. Protein deficiency is the most important factor leading to low performance of ruminants fed low quality forages. *Calliandra calothyrsus* preserved in silage is an alternative method for improving crude protein (CP) contents of feeds for sustainable ruminant production. The CP supplies N-protein for microbial protein synthesis in the rumen. Manipulation of the rumen microbial ecosystem for enhancing fiber digestibility, reducing methane (CH_4) production and improving animal performance are high priority goals for ruminant nutrition (Lopez *et al*., 2010).

The *C. calothyrsus* contains high levels of condensed tannins. Condensed tannins are polymeric proanthocyanidins, composed of flavonoid units (Bhat *et al*., 1998). Tannins have a capacity to form complexes with proteins including proteolytic enzymes, thus reducing nutrient degradation (Kamra *et al*. 2012; Jayanegara & Sofyan, 2008). The reactive nature of tannins in feeds indicates that they could be used in nutritional strategies to reduce CH_4 emissions from ruminants in tropical regions (Tiemann *et al*., 2008; Jayanegara *et al*., 2011a).

However, CH_4 is a potent greenhouse gas, which contributes to global warming (Patra, 2014; Bodas *et al*., 2012). Ruminants are considered as one of the high contributors to atmospheric pollution by enteric fermentation (Patra, 2014; Ji & Park, 2012). The CH_4 is produced normally in the rumen by methanogens, of which the major substrates such as CO_2 and H_2 are supplied by protozoa, fungi, and bacteria during fermentation of the feed. Enteric CH_4 production in ruminant has been intensively studied, and the beneficial effect of tannin to reduce CH_4 emission has also been reported by several authors (Tiemann *et al*., 2008; Jayanegara *et al*., 2009, 2011a, b, c, 2013; Patra & Saxena, 2010; Castro-Montoya *et al*., 2011). However, microbial interactions with tannins and the mechanism of CH_4 mitigation in the rumen are still unclear. Molecular approaches based on 16S rDNA of terminal restriction fragment length polymorphisms (T-RFLP) has been applied to investigate microbial diversity from different ecosystems (Liu *et al*., 1997; Blackwood *et al*., 2007) and for quantifying members of microbial communities using quantitative real rime-PCR (qPCR) (Bustin *et al*., 2009). The objective of this study was to evaluate the effectiveness of silages diet containing *C. calothyrsus* on CH_4 mitigation and microbial diversity in a rumen *in vitro* fermentation using the Hohenheim Gas Test (HGT).

MATERIALS AND METHODS

Preparation of Silage

Silage was made from our previous work and was chosen with the best quality silage (under process of publication). Grass-legumes silages were made by using wilted king grasses (*Pennisetum purpureum* hybrid) and *C. calothyrsus* (Fabaceae; red flower**)** legumes with combination of 50%:50% (w/w). Grasses and legumes were chopped to the lengths of approximately 3-5 cm. Ready available carbohydrate (10%) and inoculants of BTCC570 ($2.5x10^6$ CFU/g material) were used as silage additives. Silages were prepared by using plastic jar silos (600 g) with three replications and then incubated at room temperature (30°C) for 30 d. After 30 d, the silage was opened and before being used for fermentation, substrate was lyophilized by using a freeze dryer for 48 h, ground and then sieved through a 0.5 mm screen. For evaluation of silage quality, chemical compositions analysis such as proximate, fiber fraction, and tannin contents (AOAC, 1997; Van Soest *et al*., 1991; Makkar, 2003) and microbiology analyses (Sakamoto *et al*., 2004) were conducted.

In Vitro Rumen Fermentation

The rumen fluid was obtained from three fistulated ongole breed cattles before the morning feeding. The use of the cattle in this experiment was approved by the Animal Care and Use Committee of Bogor Agricultural University (No.01-2013 IPB). All cattles were given feed at 2% DM of body weight (230 kg) with composition of grass (*P. purpureum* hybrid) and commercial concentrate, 60% : 40%. Rumen fluid used as the source of inoculum was mixed, homogenized, filtered by using sterilized double cheesecloth and transferred to a glass flask, constantly flushed with CO_2 and kept warm in a water bath at 39 °C.

The HGT uses the protocol of Lopez *et al*. (2010) based on the method of Menke *et al*. (1979) and modification method by Castro-Montoya *et al*. (2011). The substrate was approximately 380 mg for each treatment and incubated in 100 mL-capacity glass syringes. Thirty milliliters of buffered medium consisted of double strength buffer and rumen fluid (with the ratio of 2:1) was dispensed into glass syringes and incubated in a water bath at 39 °C for 24 h. The gas production was observed every 2 h for 12 h, and finally at 24 h (2, 4, 6, 8, 10, 12, and 24 h of incubation). The net gas production was calculated by subtracting the values of the blank from that of the test syringe. For CH_4 concentration analysis, the gas was collected by using 10 mL sterilized syringes in two parts of incubation times, at 12 h and 24 h and then it was directly placed into a 5 mL of vacuum Venoject tube. The CH_4 was analyzed from mixed gas of each treatment by using GC-TCD (Shimadzu 8A). After 24 h incubation, the buffer medium was collected and divided into sterilized corning tubes for chemical analysis of pH (Cyberscan pH310 Eutech), N-NH_3 (Conway method), VFAs (GC-FID, Bruker Scion 436) and for microbial analysis of T-RFLP, qPCR, and protozoa numbers (Ogimoto & Imai, 1981). The *in vitro* organic matter digestibility (IVOMD) was calculated by following the equations, IVOMD (mg/g): 148.8 + 8.893 gas production (mL) + 0.448 CP (g/kg DM) + 0.651 total ash (g/kg DM) (Menke & Steingass, 1988).

Microbial Diversity Analyses

Microbial DNA from buffer medium of each treatment was extracted by using Genomic DNA Mini

Kit (Blood or Culture Cell) based on Buffy Coat Protocol (Geneaid) with some modifications such as addition of Proteinase K (final concentration of 2 mg/mL) and RNAse A (final concentration of 10 mg/mL), and then incubated at 60°C for 30 min. The DNA was pooled from each treatment with a total of 5 DNA samples were collected.

The DNA was amplified by using primer 6FAM-27F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'GGTTACCTTGTTACGACTT3') for bacteria (Lane, 1991) and 6FAM-Met86F (5'GCTCAGTAACACGTGG3') and Met1340R 5'CGGTGTGTGCAAGGAG3') for methanogens (Wright *et al*., 2004). Amplification of PCR reaction was performed as described previously (Sakamoto *et al*., 2004) in a total volume of 50 μL consisted of 5 μL of dissolved DNA (<1 μg), 0.5 μL of 1.25U Takara Ex Taq (Takara shuzo), 5 μL of 10x Ex Taq buffer, 4 μL of dNTP mixture (2.5 mmol/L), 10 pmol of each primer and up to 50 μL of pure distilled water. The 16S rDNAs were amplified by using a Biometra Thermocycler TGradient with the following program for bacteria (Sakamoto *et al*., 2004): 95°C for 3 min, followed by 30 cycles consisted of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1.5 min, with a final extension at 72°C for 10 min and for methanogens (Danielsson *et al*., 2012): 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 68 °C for 1 min; with a final extension at 68 °C for 7 min. Amplified DNAs were verified by electrophoresis of aliquots PCR product (5 μL) in 1.5% agarose in 1x TAE buffer. The PCR products were purified by using an Ultra Clean PCR Clean Up Kit (Mo Bio Laboratories, Inc.,). The purified 16S rDNAs were stored at -20 °C until analysis.

T-RFLP was analyzed based on the method of Sakamoto *et al*. (2004) and Danielsson *et al*. (2012) with some modifications. The purified PCR product (2 μL) was digested with four restriction enzymes consisted of 20 U of *Alu*I, *Hha*I, *Msp*I and *Rsa*I (TaKaRa Shuzo) in a total volume of 10 μL at 37 °C for 1 h. The restriction digest product (2 μL) was mixed with 8μL of Hi-Di Formamide (Applied Biosystems) and 1 μL of standard Gene Scan™ 1200 LIZ (Applied Biosystems). Each sample was denatured at 95 °C for 2 min and then immediately placed on ice. The length of T-RF was determined by using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) in GeneScan mode. T-RFs were estimated by using local method peak scan version 2.0 (Applied Biosystems). T-RFs with area peak of less than 2% of total area were excluded from the analysis. DNA fragments were resolved to one base pair by manual alignment of the standard peaks from different electropherograms. The prediction of T-RFs was performed by using the microbial diversity data base of MiCAIII (Shyu *et al*., 2007). Diversity of microbial populations was determined based on the method of diversity index Smith and Wilson evenness (*E*var) as described by Blackwood *et al*. (2007).

Quantitative real time PCR was performed as described previously (Sakamoto *et al*., 2004; Danielsson *et al*., 2012; Bustin *et al*., 2009) with some modifications by using the LightCycler3 system (Roche Diagnostic) in accordance with the manufacturer's instructions and the dsDNA-binding dye SYBR GreenI with four pairs of specific primers (Denman & McSweeney, 2006; Denman *et al*., 2007; Yu *et al*., 2005). The amplification was determined in a 20 μL final volume consisted of 10 μL of SYBR® premix ExTaq™ containing TLi RNase H plus (Takara), 0.4 μL of each specific primer (forward & reverse) contained 10 pmol of the final concentration, 7.2 μL of pure distilled water, and 2 μL of DNA sample from each treatment. The total number of bacteria in samples was determined by using *Escherichia coli* JM 109 cells as a standard. The total number of methanogens (including Methanobacteriales and Methanosarcinales) in samples was determined by using *Methanosarcina barkeri* JCM 10043^T or *Methanobrevibacter ruminantium* JCM 13430^T cells as a standard. The DNA of microbial standards was extracted by using Genomic DNA Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). A melting curve was used to determine the specificity of the PCR. Data were analyzed by using the LightCycler analysis software version 5.3 (Roche Diagnostic).

Experimental Design and Statistical Analysis

The experiment was a completely randomized design with five treatments and three replications. The treatments consisted of different levels of silages containing 50% (w/w) of *C. calothyrsus* namely K; 100% concentrate + pure tannic acid of 1 mg/mL (Merck cat. no.1.00773), R1; 25% silage + 75% concentrate, R2; 50% silage + 50% concentrate, R3; 75% silage + 25% concentrate, and R4; 100% silage. The fermentation parameters measured were total gas, CH_4, IVOMD, VFAs, pH, N-NH_3, numbers of protozoa, and microbial diversity. Data were analyzed by using ANOVA with SPSS 16 for windows. Significant effects of each treatment were further analyzed by using the least significant difference by Duncan Test ($P<0.01$), except microbial diversity data were analyzed descriptively.

RESULTS AND DISCUSSION

Rumen Fermentation and Methane Mitigation

The chemical compositions of feed treatments are presented in Table 1. The increasing level of silage

Table 1. Chemical composition of dietary treatments

Composition g/kg	Treatment				
	K	R1	R2	R3	R4
DM	915.9	910.6	900.3	903.9	889.3
Organic matter	884.6	890.0	886.7	904.4	908.4
Crude protein	180.5	179.4	179.3	176.4	177.1
Neutral detergent fiber	411.4	444.2	480.0	508.7	548.2
Acid detergent fiber	213.1	267.0	323.3	375.1	435.2
Acid detergent lignin	131.0	161.5	193.5	222.7	256.8
Hemicellulose	198.3	177.2	156.7	133.6	113.0
Cellulose	65.5	86.5	108.4	128.7	152.0
Total phenols	NA	17.8	35.6	53.3	71.1
Total tannin	*	12.1	24.2	36.2	48.3

Note: *standard tannic acid, NA: Not available, K: 100% concentrate + pure tannic acid of 1 mg/mL, R1: 25% silage + 75% concentrate, R2: 50% silage + 50% concentrate, R3: 75% silage + 25% concentrate, R4: 100% silage.

increased NDF, ADF, cellulose, lignin and tannin and decreased EE and hemicellulose contents in the diet. The patterns of gas production kinetics from all treatments are shown in Figure 1. Several parameters of rumen fermentation system can be used to determine the quality of ruminant feed such as gas production, IVOMD and CH_4 concentration. Banik *et al*. (2013) identified that CH_4 production had a positive correlation with some fermentation parameters such as gas pressure and acetate : propionate ratio.

The fermentation characteristics of gas production, IVOMD and CH_4 concentration are shown in Table 2. Increasing levels of silage reduced gas production, IVOMD and CH_4 concentration. The total gas production of K and R1 were higher compared with R2, R3, and R4. Meanwhile, high total tannin contents in R2, R3, and R4 tended to inhibit microbial activity in the rumen to degrade the substrates that finally reduced total gas production. The inhibition effects of tannin on in vitro gas production has also been observed by several authors (Jayanegara *et al*., 2011a, b, c; Jayanegara & Sofyan, 2008; Wina *et al*., 2010). Methane concentration was influenced by the use of silage containing *C. calothyrsus*. The CH_4 in R4 was significantly lower (P<00.1) than K, R1, and R2 treatments. The use of silage up to 100% produced the lowest concentration of CH_4, but had negative effect on IVOMD. In this study the reduction in methane was 26.8% greater than that reported by Jayanegara *et al*. (2011a), who showed that *C. calothyrsus* powder containing 81g/kg total tannin produced 112 mL CH_4/L total gas in HGT. These observations are consistent with notion that feed containing tannin can be used to reduce enteric CH_4 but may have negative effects on the OM digestibility (Jayanegara *et al*., 2011a; Tiemann *et al*., 2008). The IVOMD values of R2, R3, and R4 were lower than K and R1. In this case, the increase in tannin contents in silages decreased rumen microbial activity. Kamra *et al*. (2012) described that tannins had mechanism to inhibit methanogenesis either directly or indirectly.

Rumen metabolite profiles consisting of pH, N-NH_3 and VFAs are shown in Table 3. The pH values varied in all treatments and ranged between 6.36 and 6.71. These values are within the normal range for growth of cellulolytic bacteria. The pH value of R4 was higher (P<0.01) than K, R1, and R2. The concentration of N-NH_3 was the lowest in the K (P<0.01) compared with other treatments. The lowest level of ammonia production in the K treatment was related to the highest level of tannin. Increased N-NH_3 concentration indicates higher protein degradation by rumen microbes. The use of pure tannin in K treatment showed that the protein were bound by tannin and protected from rumen microbial degradation. Rumen microbial activity affects the metabolites profile, mainly VFAs. The use of silages in the diets significantly increased the percentage of C2 volatile fatty acids and decreased C3, but C4 and C5 fatty acids were unchanged (Table 3). The percentage of C2 in R2, R3, and R4 were significantly higher (P<0.01) than K and R1 treatments.

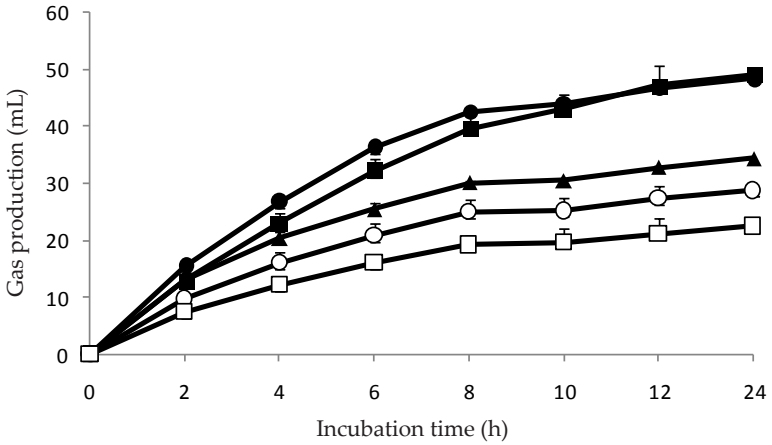

Figure 1. Kinetics of gas production in HGT fermentation system. (■) K; 100% concentrate + pure tannic acid of 1 mg/ml, (●) R1; 25% silage + 75% concentrate, (▲) R2; 50% silage + 50% concentrate, (○) R3; 75% silage + 25% concentrate, and (□) R4; 100% silage.

Table 2. Organic matter digestibility and gas production

Treatment	IVOMD (mg/g)	Total gas (mL)	CH_4 (mL)	CH_4/total gas (mL/L)
K	693.53±31.13^{b}	59.50±3.50^{b}	6.84±0.72^{c}	114.81± 5.99^{b}
R1	663.49± 6.79^{b}	56.17±0.76^{b}	7.76±0.37^{c}	138.12± 5.34^{b}
R2	539.20±78.33^{a}	42.17±8.81^{a}	5.23±1.55^{bc}	122.33±13.14^{b}
R3	478.63±20.38^{a}	35.50±2.29^{a}	3.92±0.65^{ab}	109.83±11.53^{ab}
R4	420.59±25.15^{a}	29.00±2.00^{a}	2.41±0.77^{a}	82.01±18.68^{a}

Note: IVOMD: *in vitro* organic matter digestibility. K: 100% concentrate + pure tannic acid of 1 mg/ml, R1: 25% silage + 75% concentrate, R2: 50% silage + 50% concentrate, R3: 75% silage + 25% concentrate, and R4: 100% silage. Means in the same column with different superscript differ significantly (P<0.01).

Table 3. Profile of rumen fermentation

Treatment	pH	N-NH_3 (mmol/L)	VFAs (% molar proportion)						C2:C3
			C2	C3	C4	isoC4	C5	isoC5	
K	6.36±0.08^{a}	29.06±0.53^{a}	60.48±0.47^{a}	24.97±0.73^{b}	11.30±0.19^{a}	1.22±0.04^{a}	0.70±0.03^{a}	1.33±0.06^{a}	2.42±0.09^{a}
R1	6.43±0.03^{ab}	49.42±1.52^{c}	60.61±0.96^{a}	23.42±0.84^{ab}	11.46±0.94^{a}	1.60±0.06^{a}	1.02±0.35^{b}	1.89±0.37^{b}	2.59±0.06^{ab}
R2	6.52±0.05^{bc}	43.69±0.30^{bc}	61.43±0.31^{bc}	23.29±0.55^{ab}	11.06±0.38^{a}	1.39±0.03^{a}	1.04±0.11^{b}	1.80±0.13^{ab}	2.64±0.06^{ab}
R3	6.60±0.03^{cd}	42.31±4.31^{b}	62.43±0.83^{bc}	22.43±0.50^{a}	10.82±0.33^{a}	1.43±0.11^{a}	1.07±0.05^{b}	1.82±0.05^{ab}	2.79±0.10^{b}
R4	6.71±0.04^{d}	37.64±2.14^{b}	62.99±0.79^{c}	22.32±0.74^{a}	10.41±0.22^{a}	1.41±0.06^{a}	1.06±0.10^{b}	1.8±0.12^{ab}	2.82±0.12^{b}

Note: C2: acetate, C3: propionate, C4: butyrate, C5: valerate. K: 100% concentrate + pure tannic acid of 1 mg/ml, R1: 25% silage + 75% concentrate, R2: 50% silage + 50% concentrate, R3: 75% silage + 25% concentrate, and R4: 100% silage. Means in the same column with different superscript differ significantly (P<0.01).

R3 and R4 had lower C3 production compared to K treatment. High yield of acetate and low propionate generally produced high concentration of CH_4, but in this result showed different patterns. This profile did not directly affect the production of CH_4 meaning that H_2 was not optimally used by methanogens. These finding suggested that methanogenesis was inhibited by tannin contained in silages *C. calothyrsus*. High concentration of polyphenolic in the feed can inhibit digestibility, absorption and reduce CH_4 production and energy loss. In this study, R1 to R2 with combination of 50 to 75% and 25 to 50% (concentrate and silage) had adequate tannin to reduce enteric CH_4, but still had negative effect on reducing IVOMD.

Microbial Diversity

Diversity index and microbial population are shown in Table 4. The increasing level of silage in the diets decreased the diversity index of bacteria and protozoa populations. R4 showed the lowest *E*var value of bacteria compared to the other treatments, while R1 had the highest. These results implied that at lower concentrations of tannins the diversity was not affected, but at higher concentration of polyphenolic, there was a consistent decrease. Inclusion of polyethyleneglicol to neutralize the tannin effect may help resolve this issue. Protozoa population of R4 was the lowest amongst the treatments. The use of pure tannic acid did not significantly affect protozoa number in R2 and R3. The treatments might have closely similar function in reducing the numbers of protozoa. Ranilla *et al*. (2007) described that the increase in the population of protozoa such *Entodinium caudatum* stimulated the production of CH_4. Figure 2a and b showed the pattern of changes in microbial community. The T-RFLP analysis indicated the population of 17 T-RFs of bacterial phylotype where four uncultured bacteria were found (Figure 2a). The increase in the level of silage had tendency to reduce the population of *Propionibacterium acidipropionici, Prevotella multiformis, Desulfovibrio oxamicus, Syntrophomonas erecta, Desulfovibrio* sp., *Peptostreptococcus* sp., *Cellulophaga* sp. and uncultured rumen bacteria. The decreased of these bacteria had positive correlation with the VFA production, especially C3. *Desulfovibrio oxamicus*, *Cellulophaga* sp., uncultured proteobacterium, uncultured *Spirochaetes* population and two T-RFs of 6 and 13 bp indicating they were tolerant to high level of tannin-containing silages used in the fermentation system. McSweeney *et al*. (2001) reported that proteolytic bacteria were present in relatively high number and tolerant to highly tanniniferous diet.

Furthermore, the methanogenic community analysis identified 8 predominant T-RFs including 2 T-RFs identified as unknown fragment (137 and 150 bp) (Figure 2b). Nine methanogens and archaea were found culturable identity based on GenBank data base, while the other of 10 were unculturable. The culturable methanogens consisted of *Methanobrevibacter ruminantium, Methanoplanus petrolearius, Methanothermobacter thermoflexus, Methanobacterium subterraneum, Methanothermococcus* sp., *Methanotorris igneus*, *Methanobacterium* sp., *Methanococcus vannielii,* and *Methanocaldococcus vulcanius.* Increased level of silages tended to decrease the population of

Table 4. Diversity index and numbers of rumen microbes

Target microbes	Treatment				
	K	R1	R2	R3	R4
*E*var; Bacteria*	0.82	0.88	0.81	0.79	0.67
Methanogens*	0.55	0.52	0.49	0.51	0.50
Total bacteria**	$3.24x10^{11}$	$1.74x10^{11}$	$2.13x10^{11}$	$3.91x10^{11}$	$2.20x10^{11}$
Total methanogens**	$7.51\ x10^{9}$	$6.09x10^{9}$	$5.87x10^{9}$	$6.99x10^{9}$	$6.03x10^{9}$
Methanobacteriales (%TM)	11.17	17.79	16.55	13.29	12.17
Methanosarcinales (%TM)	0.021	0.018	0.019	0.023	0.011
Protozoa (Log10/mL)	5.90±0.07^{b}	6.17±0.09^{c}	6.11±0.08^{bc}	5.95±0.08^{bc}	5.60±0.09^{a}

Note: *5% threshold standardized, **copy number of 16S rDNAs (qPCR), TM: total methanogens. K: 100% concentrate + pure tannic acid of 1 mg/ml, R1: 25% silage + 75% concentrate, R2: 50% silage + 50% concentrate, R3: 75% silage + 25% concentrate, and R4: 100% silage. Means in the same row with different superscript differ significantly (P<0.01).

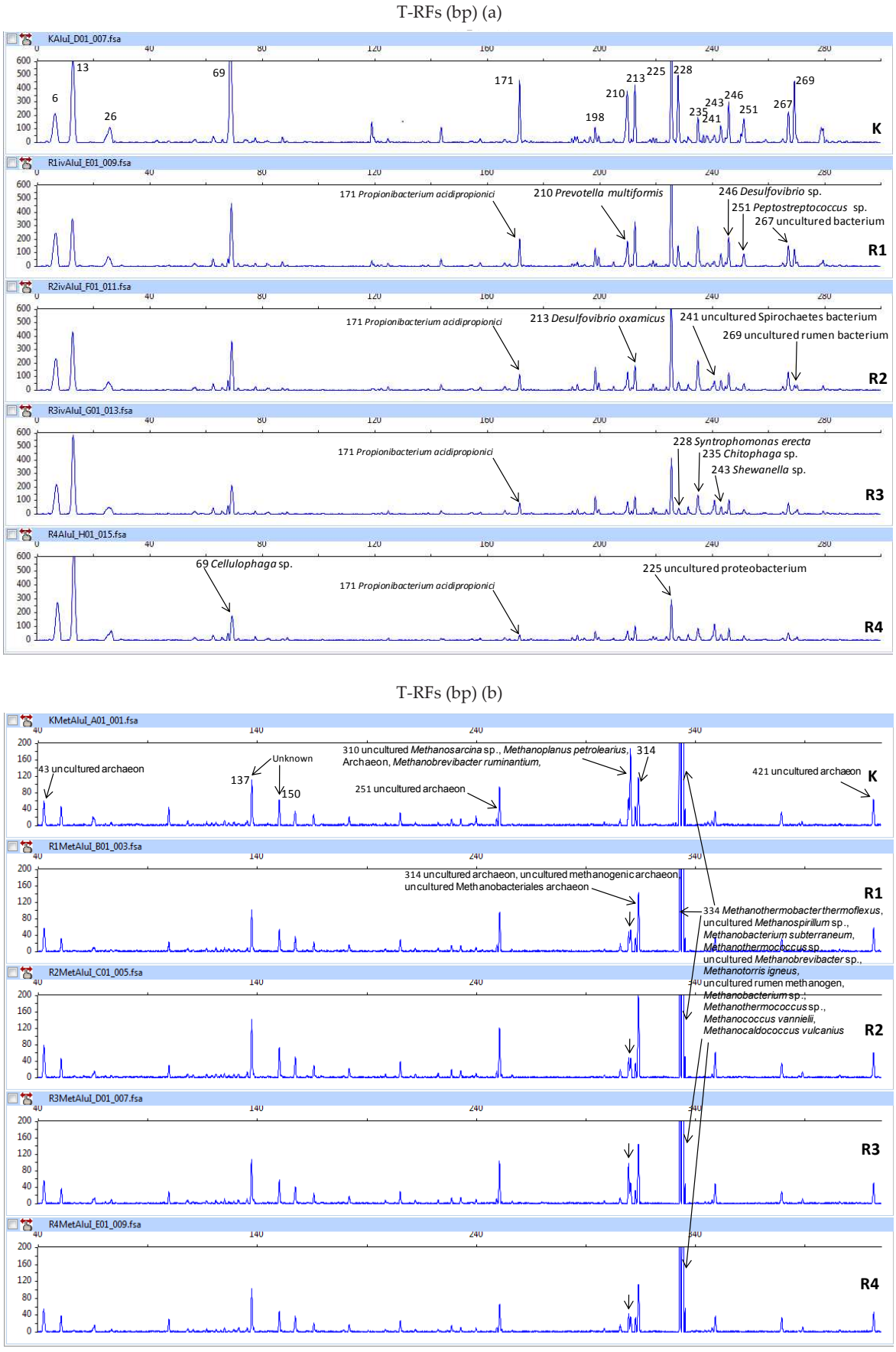

Figure 2. T-RFLP 16S rDNAs profile after digested by AluI; (a) bacteria and (b) methanogens. K: 100% concentrate + pure tannic acid of 1 mg/ml, R1: 25% silage + 75% concentrate, R2: 50% silage + 50% concentrate, R3: 75% silage + 25% concentrate, and R4: 100% silage.

Methanobrevibacter ruminantium, Methanoplanus petrolearius, uncultured *Methanosarcina* sp., and archaea. Community of methanogens in 334 bp was consistently dominant from all treatments (Figure 2b). This result had close similarity with the results reported by Danielsson *et al*. (2012). Addition of silages directly inhibited the methanogenesis, while indirectly decreased the activity of bacteria and protozoa. The inhibitory effect of tannin on rumen methanogenesis is related to the direct effects on methanogens, and indirectly through a depression of protozoa associated with CH_4 production and cellulolytic bacteria on fiber digestion (Kamra *et al*., 2012).

The qPCR analysis detected total methanogens and Methanobacterales population decreased consistently with the increasing level of silage, except for total bacteria. The decrease in microbial diversity index is not always followed by the reduction of population. This fact is occurred on bacteria, but does not occur in methanogens (Brulc *et al*., 2011; Belanche *et al*., 2012). In this experiment, the diversity of methanogens was consistently stable, but the population declined (Table 4). These results were in agreement with Singh *et al*. (2012) who reported that Methanobacteriales was a common population in the rumen and positively correlated with CH_4 production. This includes the type of hydrogenothrophic methanogens which capable of using CO_2 and H_2 to produce CH_4. Methanobacteriales population reduced consistently with the increasing level of silage, and then decreased CH_4 production (Table 2). *Methanobrevibacter ruminantium* was included as Methanobacteriales order whose population decreased with the increasing levels of silage (Table 4). The activity was inhibited because of the reduced protozoa population which was associated with tannin content. Protozoa and methanogens were known to have high association in the rumen through endosymbiont mechanisms.

CONCLUSION

The use of silage containing *C. calothyrsus in vitro* fermentation system reduces the enteric CH_4 production. The increasing level of silages reduces CH_4, gas production, IVOMD value, $N\text{-}NH_3$ concentration, bacterial diversity index, and protozoal population. Methanobacteriales population has consistently abundance and positively correlated with CH_4 production. Addition of 25% to 50% silage diets has been effective in reducing enteric methane production but has negative effect on decreasing bacterial diversity index and OM digestibility.

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REFERENCES

AOAC. 1997. Official Methods of Analysis. Association of Official Analytical Chemists, Arlington, VA, USA.

Banik, B. K., Z. Durmic, W. Erskine, K. Ghamkhar, & C. Revell. 2013. In vitro ruminal fermentation characteristics and methane production differ in selected key pasture species in Australia. Crop & Pasture Science. 64:935–942. http://dx.doi.org/10.1071/CP13149

Belanche, A., G. de la Fuente, E. Pinloche, C. J. Newbold, & J. Balcells. 2012. Effect of diet and absence of protozoa on the rumen microbial community and on the representativeness of bacterial fractions used in the determination of microbial protein synthesis. J. Anim. Sci. 90:3924–3936. http://dx.doi.org/10.2527/jas.2011-4802

Bhat, T. K., B. Singh, & O. P. Sharma. 1998. Microbial degradation of tannins–a current perspective. Biodegradation. 9:343–357. http://dx.doi.org/10.1023/A:1008397506963

Blackwood, C. B., D. Hudleston, D. R. Zak, & J. S. Buyer. 2007. Interpreting ecological diversity indices applied to terminal restriction fragment length polymorphism data: insights from simulated microbial communities. Appl. Environ. Microbiol. 73:5276–5283. http://dx.doi.org/10.1128/AEM.00514-07

Bodas, R., N. Prieto, R. Garcia-Gonzalez, S. Andres, F. J. Giraldez, & S. Lopez. 2012. Manipulation of rumen fermentation and methane production with plant secondary metabolites. Anim. Feed Sci. Technol. 176:78–93. http://dx.doi.org/10.1016/j.anifeedsci.2012.07.010

Brulc, J. M., C. J. Yeoman, M. K. Wilson, M. E. B. Miller, P. Jeraldo, S. Jindou, N. Goldenfeld, H. J. Flint, R. Lamed, I. Borovok, M. Vodovnik, K. E. Nelson, E. A. Bayer, & B. A. White. 2011. Cellulosomics, a gene-centric approach to investigating the intraspecific diversity and adaptation of Ruminococcus flavefaciens within the rumen. PLoS ONE 6:e25329. http://dx.doi.org/10.1371/journal.pone.0025329

Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R Mueller, T. Nolan, M. W. Pfaffi, G. L. Shipley, J. Vandesompele, & C. T. Wittwer. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry. 55:611–622. http://dx.doi.org/10.1373/clinchem.2008.112797

Castro-Montoya, J. M., H. P. S. Makkar, & K. Becker. 2011. Chemical composition of rumen microbial fraction and fermentation parameters as affected by tannin and saponins using an in vitro rumen fermentation. Can. J. Anim. Sci. 91:433–448. http://dx.doi.org/10.4141/cjas2010-028

Danielsson, R., A. Schnurer, V. Arthurson, & J. Bertilsson. 2012. Methanogenic population and CH_4 production in Swedish dairy cows fed different levels of forage. Appl. Environ. Microbiol. 78:6172-6179. http://dx.doi.org/10.1128/AEM.00675-12

Denman, S. E. & C. S. McSweeney. 2006. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. FEMS Microbiol. Ecol. 58:572–582. http://dx.doi.org/10.1111/j.1574-6941.2006.00190.x

Denman, S. E., N. W. Tomkins, & C. S. McSweeney. 2007. Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. FEMS Microbiol. Ecol. 62:313–322. http://dx.doi.org/10.1111/j.1574-6941.2007.00394.x

Jayanegara, A., E. Wina, C. R. Soliva, S. Marquardt, M. Kreuzer, & F. Leiber. 2011a. Dependence of forage quality and methanogenic potential of tropical plants on their phenolic fractions as determined by principal component analysis. Anim. Feed Sci. Technol. 163:231–243. http://dx.doi.org/10.1016/j.anifeedsci.2010.11.009

Jayanegara, A., F. Leiber, & M. Kreuzer. 2011b. Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from *in vivo* and *in vitro* experiments. J. Anim. Physiol. Anim. Nutr. 96:365–375. http://dx.doi.org/10.1111/j.1439-0396.2011.01172.x

Jayanegara, A., S. Marquardt, M. Kreuzer, & F. Leiber. 2011c. Nutrient and energy content, in vitro ruminal fermentation characteristics and methanogenic potential of alpine forage plant species during early summer. J. Sci. Food Agric. 91:1863–1870. http://dx.doi.org/10.1002/jsfa.439

Jayanegara, A. & A. Sofyan. 2008. Penentuan aktifitas aktivitas biologis tannin beberapa hijauan secara in vitro menggunakan 'Hoheinheim Gas Test' dengan polietilen glikol sebagai determinan. Med. Pet. 31:44–52.

Jayanegara, A., N. Togtokhbayar, H. P. S. Makkar, & K. Becker. 2009. Tannins determined by various methods as predictors of methane production reduction potential of plants by an in vitro rumen fermentation system. Anim. Feed Sci. Technol. 150:230–237. http://dx.doi.org/10.1016/j.anifeedsci.2008.10.011

Jayanegara, A., S. Marquardt, E. Wina, M. Kreuzer, & F. Leiber. 2013. In vitro indications for favourable non-additive effects on ruminal methane mitigation between high-phenolic and high-quality forages. British Journal of Nutrition. 109:615–622. http://dx.doi.org/10.1017/S0007114512001742

Ji, E. S. & K. Park. 2012. Methane and nitrous oxide emissions from livestock agriculture in 16 local administrative districts of Korea. Asian-Aust. J. Anim. Sci. 25:1768–1774. http://dx.doi.org/10.5713/ajas.2012.12418

Kamra, D. N., M. Pawar, & B. Singh. 2012. Effect of plant secondary metabolites on rumen methanogens and methane emissions by ruminants. In: A. K. Patra (Ed). Dietary Phytochemicals and Microbes. Spinger Sciences Business Media, Dordrecht. p.351–370. http://dx.doi.org/10.1007/978-94-007-3926-0_12

Lane, D. J. 1991. 16S/23S rRNA sequencing. In: E. Stackebrandt & M. Goodfellow (eds). Nucleic Acid Techniques in Bacterial Systematics. Chichester: Wiley. pp.115–175.

Liu, W. T., T. L. Marsh, H. Cheng, & L. J. Forney. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63:4516–4522.

Lopez, S., H. P. S. Makkar, & C. R. Solivia. 2010. Screening plants and plant products for methane inhibitors. In: P. E.Vercoe, H. P. S. Makkar, & A. C. Schlink (Eds). In Vitro Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies. Spinger Science Business Media B.V. Dordrecht. p.191–232. http://dx.doi.org/10.1007/978-90-481-3297-3_10

Makkar, H. P. S. 2003. Quantification of Tannins in Tree and Shrub Foliage: A Laboratory Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands. http://dx.doi.org/10.1007/978-94-017-0273-7

McSweeney, C. S., B. Palmer, D. M. McNeill, & D. O. Krause. 2001. Microbial interactions with tannins: nutritional consequences for ruminants. Anim. Feed Sci. Technol. 91:83–93. http://dx.doi.org/10.1016/S0377-8401(01)00232-2

Menke, K. H. & H. Steingass. 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim. Res. Dev. 28:7–55.

Menke, K. H., L. Raab, A. Salewski, H. Steingass, D. Fritz, & W. Schneider. 1979. The estimation of the digestibility and metabolizable energy content of ruminant feeding-stuff from the gas production when they are incubated with rumen liquor *in vitro*. J. Agric. Sci. 93:217–222. http://dx.doi.org/10.1017/S0021859600086305

Ogimoto, K. & S. Imai. 1981. Atlas of Rumen Microbiology. Japan Sci. Soc. Press, Tokyo.

Patra, A. K. 2014. Trends and projected estimates of GHG emissions from Indian livestock in comparisons with GHG emissions from world and developing countries. Asian-Aust. J. Anim. Sci. 27:592–599. http://dx.doi.org/10.5713/ajas.2013.13342

Patra, A. K. 2014. Trends and Projected Estimates of GHG Emissions from Indian Livestock in Comparisons with GHG Emissions from World and Developing Countries. Asian-Aust. J. Anim. Sci. 27:592–599. http://dx.doi.org/10.5713/ajas.2013.13342

Ranilla, M. J., J. P. Jouany, & D. P. Morgavi. 2007. Methane production and substrate degradation by rumen microbial communities containing single protozoal species in vitro. Letters in Appl. Microbiol. 45:675–680. http://dx.doi.org/10.1111/j.1472-765X.2007.02251.x

Sakamoto, M., Y. Huang, M. Ohnishi, M. Umeda, I. Ishikawa, & Y. Benno. 2004. Changes in oral microbial profiles after periodontal treatment as determined by molecular analysis of 16 rRNA genes. J. Med. Microbiol. 53:563–571. http://dx.doi.org/10.1099/jmm.0.45576-0

Shyu, C., T. Soule, S. J. Bent, J. A. Foster, & L. J. Forney. 2007. MiCA: a web-based tool for the analysis of microbial communities based on terminal-restriction fragment length polymorphisms of 16S and 18S rRNA genes. Microb. Ecol. 53:562–570. http://dx.doi.org/10.1007/s00248-006-9106-0

Singh, K. M., A. K. Tripathi, P. R. Pandya, S. Parnerkar, D. N. Rank, R. K. Kothari, & C. G. Joshi. 2012. Methanogen diversity in the rumen of Indian surti buffalo (*Bubalus bubalis*), assessed by 16S rDNA analysis. Research in Veterinary Science. 92:451–455. http://dx.doi.org/10.1016/j.rvsc.2011.03.022

Tiemann, T. T., C. E. Lascano, H. R. Wettstein, A. C Mayer, M. Kreuzer, & H. D. Hess. 2008. Effect of the tropical tannin-rich shrub legumes *Calliandra calothyrsus* and *Flemingia macrophylla* on methane emission and nitrogen and energy balance in growing lambs. Animal. 2:790–799. http://dx.doi.org/10.1017/S1751731108001791

Van Soest, P. J., J. B. Robertson, & B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583–3597. http://dx.doi.org/10.3168/jds.S0022-0302(91)78551-2

Wina, E., I. W. R. Susana, & B. Tangendjaja. 2010. Biological activity of tannins from *Acacia mangium* bark extracted by different solvents. Med. Pet. 33: 103–107. http://dx.doi.org/10.5398/medpet.2010.33.2.103

Wright, A. D., A. J. Williams, B. Winder, C. T. Christophersen, S. L. Rodgers, & K. D. Smith. 2004. Molecular diversity of rumen methanogens from sheep in Western Australia. Appl. Environ. Microbiol. 70:1263–1270. http://dx.doi.org/10.1128/AEM.70.3.1263-1270.2004

Yu, Y., C. Lee, J. Kim, & S. Hwang. 2005. Group-Specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol. and Bioengineering. 89:670–679. http://dx.doi.org/10.1002/bit.20347