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$_4$ concentration, 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 calothyrsus*, 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$_4$, 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 Methanobacteriales. 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 dan valerate) 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 kecerunan 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. Calliandira 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 \((\text{CH}_4)\) 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 & Soyfan, 2008). The reactive nature of tannins in feeds indicates that they could be used in nutritional strategies to reduce \(\text{CH}_4\) emissions from ruminants in tropical regions (Tiemann et al., 2008; Jayanegara et al., 2011a).

However, \(\text{CH}_4\) 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 \(\text{CH}_4\) is produced normally in the rumen by methanogens, of which the major substrates such as \(\text{CO}_2\) and \(\text{H}_2\) are supplied by protozoa, fungi, and bacteria during fermentation of the feed. Enteric \(\text{CH}_4\) production in ruminant has been intensively studied, and the beneficial effect of tannin to reduce \(\text{CH}_4\) 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 \(\text{CH}_4\) 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 time-PCR (qPCR) (Bustin et al., 2009). The objective of this study was to evaluate the effectiveness of silages diet containing C. calothyrsus on \(\text{CH}_4\) 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 (\textit{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 inclusions 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.

\textbf{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 (\textit{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 \(\text{CO}_2\) 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. 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 \(\text{CH}_4\) 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 \(\text{CH}_4\) 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 cornings 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 number (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).

\textbf{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′AGAGTTGTGATCCTGCTCAG3′) and 1492R (5′GGTTACCTTGTTACGACTT3′) for bacteria (Lane, 1991) and 6FAM-Met86F (5′GCTCAAGTAACAGCTTG3′) 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) in a to-

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 Alul, Hhal, MspI and RsaI (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 GeneScan™ 1200 LIZ (Applied Biosystems). Each sample was dena-
tured 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-

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          | K   | R1  | R2  | R3  | R4  |
|----------------------|-----|-----|-----|-----|-----|
| 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 | 375.3 | 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        | 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. 

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% concen-

RESULTS AND DISCUSSION

Rumen Fermentation and Methane Mitigation

The chemical compositions of feed treatments are presented in Table 1. The increasing level of 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 CH₄ concentration. Banik et al. (2013) identified that CH₄ 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 CH₄ concentration are shown in Table 2. Increasing levels of silage reduced gas production, IVOMD and CH₄ 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 CH₄ in R4 was significantly lower (P<0.01) than K, R1, and R2 treatments. The use of silage up to 100% produced the lowest concentration of CH₄, 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 CH₄/L total gas in HGT. These observations are consistent with notion that feed containing tannin can be used to reduce enteric CH₄ 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-NH₃, 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 cellulyolitic bacteria. The pH value of R4 was higher (P<0.01) than K, R1, and R2. The concentration of N-NH₃ 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-NH₃ 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) | CH₄ (mL) | CH₄/total gas (mL/L) |
|-----------|--------------|----------------|----------|----------------------|
| K         | 693.53±31.13b | 59.50±3.50c     | 6.84±0.72c | 114.81± 5.99b        |
| R1        | 663.49± 6.79b | 56.17±0.76b     | 7.76±0.37b | 138.12± 5.34b        |
| R2        | 539.20±78.33a | 42.17±8.81a     | 5.23±1.59a | 122.33±13.14b        |
| R3        | 478.63±20.38a | 35.50±2.29a     | 3.92±0.65ab| 109.83±11.53ab       |
| R4        | 420.59±25.15a | 29.00±2.00a     | 2.41±0.77a | 82.01±18.68a         |

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).
R3 and R4 had lower C3 production compared to K treatment. High yield of acetate and low propionate generally produced high concentration of CH4, but in this result showed different patterns. This profile did not directly affect the production of CH4, meaning that H2 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 CH4 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 CH4, 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 Evar 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 polyethylenealgel 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 CH4. 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, and 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 vannielii*, *Methanococcus vulcanius*, *Methanobrevibacter smithii*, *Methanobrevibacter bryantii*, *Methanococcus maripaludis*, and *Methanocellulococcus vulgaris*. Increased level of silages tended to decrease the population of

### Table 3. Profile of rumen fermentation

| Treatment | pH   | N-NH3 (mmol/L) | VFAs (% molar proportion) | C2   | C3   | C4   | isoC4 | C5   | isoC5 | C2-C3 |
|-----------|------|----------------|---------------------------|------|------|------|-------|------|-------|-------|
| K         | 6.36±0.08a | 29.06±0.53a | 60.48±0.47b | 24.97±0.73b | 11.30±0.19b | 1.22±0.04c | 0.70±0.03c | 1.30±0.06c | 2.42±0.09c |
| R1        | 6.43±0.03b | 49.42±1.52b | 60.61±0.96b | 23.42±0.84b | 11.46±0.94b | 1.60±0.06b | 1.02±0.35b | 1.89±0.37b | 2.59±0.06b |
| R2        | 6.52±0.05c | 43.69±0.30c | 61.43±0.31c | 23.29±0.55c | 11.06±0.38c | 1.39±0.03c | 1.04±0.11c | 1.80±0.13c | 2.64±0.06c |
| R3        | 6.60±0.03d | 42.31±4.31d | 62.43±0.83d | 22.43±0.50d | 10.82±0.33d | 1.43±0.11d | 1.07±0.05d | 1.82±0.05d | 2.79±0.10d |
| R4        | 6.71±0.04e | 37.64±2.14e | 62.99±0.79e | 22.32±0.74e | 10.41±0.22e | 1.41±0.06e | 1.06±0.10e | 1.86±0.12e | 2.82±0.12e |

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 column 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 CH₄ production and cellulytic bacteria on fiber digestion (Kamra et al., 2012).

The qPCR analysis detected total methanogens and Methanobacteriales 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 CH₄ production. This includes the type of hydrogenotrophic methanogens which capable of using CO₂ and H₂ to produce CH₄. Methanobacteriales population reduced consistently with the increasing level of silage, and then decreased CH₄ 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 CH₄ production. The increasing level of silages reduces CH₄ gas production, IVOMD value, N-NH₃ concentration, bacterial diversity index, and protozoal population. Methanobacterales population has consistently abundance and positively correlated with CH₄ 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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