



## Fermentation Characteristics and Microbial Diversity of Tropical Grass-legumes Silages

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**ABSTRACT:** *Calliandra calothyrsus* preserved in silage is an alternative method for improving the crude protein content of feeds for sustainable ruminant production. The aim of this research was to evaluate the quality of silage which contained different levels of *C. calothyrsus* by examining the fermentation characteristics and microbial diversity. Silage was made in a completely randomized design consisting of five treatments with three replications *i.e.*: R0, *Pennisetum purpureum* 100%; R1, *P. purpureum* 75%+*C. calothyrsus* 25%; R2, *P. purpureum* 50%+*C. calothyrsus* 50%; R3, *P. purpureum* 25%+*C. calothyrsus* 75%; and R4, *C. calothyrsus* 100%. All silages were prepared using plastic jar silos (600 g) and incubated at room temperature for 30 days. Silages were analyzed for fermentation characteristics and microbial diversity. Increased levels of *C. calothyrsus* in silage had a significant effect ( $p < 0.01$ ) on the fermentation characteristics. The microbial diversity index decreased and activity was inhibited with increasing levels of *C. calothyrsus*. The microbial community indicated that there was a population of *Lactobacillus plantarum*, *L. casei*, *L. brevis*, *Lactococcus lactis*, *Chryseobacterium* sp., and uncultured bacteria. The result confirmed that silage with a combination of grass and *C. calothyrsus* had good fermentation characteristics and microbial communities were dominated by *L. plantarum*. (**Key Words:** *Calliandra calothyrsus*, Silage, Fermentation Characteristics, Diversity, *Lactobacillus plantarum*)

### INTRODUCTION

*Calliandra calothyrsus* is a tropical legume used for ruminant feed, which contains 17% to 30% crude protein (CP), (Norton, 1994; Hess et al., 2006; Jayanegara et al., 2011a). Crude protein supplies N for microbial protein

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synthesis in the rumen. Protein deficiency is the most common factor that leads to poor performance in ruminants, due to the animals being fed forages of low quality (Hess et al., 2006). Feed with a high protein content such as *C. calothyrsus* should be preserved using suitable methods; the method used depends on geographical location. Recently, for tropical regions, including Indonesia, silage has been used as a method for preserving forages. Ensiling is a natural fermentation process where lactic acid bacteria (LAB) convert water soluble carbohydrates (WSC) into organic acids, mainly lactic acid (McDonald et al., 1991; Lynch et al., 2012). The dominance of homofermentative LAB is preferable, as rapid acidification occurs, which decreases pH to inhibit the growth of undesirable microorganisms and reduces nutritional loss in the ensiling process (McDonald et al., 1991).

The other benefit of *C. calothyrsus* is its tannin concentration of approximately 8.1% may reduce enteric

CH<sub>4</sub> (Jayaneegara et al., 2011a). Feed containing tannin seems promising as a nutritional strategy to reduce CH<sub>4</sub> emissions from ruminants in the tropics (Tiemann et al., 2008). The CH<sub>4</sub> is a potent greenhouse gas that contributes to global warming (Bodas et al., 2012; Patra et al., 2012), and enteric rumen fermentation contributes to atmospheric pollution (Moss et al., 2000).

However, legumes that have a low WSC content and high buffer capacity do not produce good quality silage (McDonald et al., 1991; Norton, 1994). Combination grasses and legumes are an alternative solution to the success of the ensiling process. Successful ensiling can be evaluated by determining the relationships between fermentation characteristics and microbial diversity in silages. Fermentation product and microbial community during ensiling in several types of ruminant forages has been reported by Li and Nishino (2013), but information about tropical forages, especially *C. calothyrsus*, is still limited. Analysis of the microbial community based on 16S rDNA is suitable for characterizing microbial diversity (Liu et al., 1997; Kaplan et al., 2001). The objective of this research was to evaluate the quality of silage containing different levels of *C. calothyrsus* by assessing fermentation characteristics and microbial diversity.

## MATERIALS AND METHODS

### Ensiling

Silage was made using wilted king grasses (*Pennisetum purpureum* hybrid) and *C. calothyrsus* (Fabaceae; red flower) legumes. The grasses were provided by the Plant Collection of the Research Center for Biotechnology. Legumes were collected from the Agrostology Field Laboratory of Bogor Agricultural University. Grasses and legumes were chopped to lengths of approximately 5 cm. Ready available carbohydrate (10%) and inoculants of *Lactobacillus plantarum* BTCC570 ( $2.5 \times 10^6$  cfu/g material) were used as silage additives. Silage was made with a completely randomized design, and consisted of five treatments with three replications *i.e.*: R0, *P. purpureum* 100%; R1, *P. purpureum* 75%+*C. calothyrsus* 25%, R2, *P. purpureum* 50%+*C. calothyrsus* 50%, R3, *P. purpureum* 25%+*C. calothyrsus* 75%, and R4, *C. calothyrsus* 100%. All silages were prepared using plastic jar silos to laboratory scale (600 g) and then incubated at room temperature ( $\pm 30^\circ\text{C}$ ) for 30 days.

### Silage sample preparation

After 30 days, silages were opened and divided into two parts; one part was oven-dried and milled to pass through a 0.5 to 1 mm aperture sieve for proximate, fiber fraction, total phenol (TP), and total tannin (TT) analyses. The second part was used for the extraction of silage juice for

detecting the pH and LAB population on the day of silo opening; the rest was stored at  $-20^\circ\text{C}$  for measuring the concentration of lactic acid, WSC, volatile fatty acids (VFA), ammonia nitrogen (NH<sub>3</sub>-N), and for microbial diversity analysis. Silage juice was made using a blender, with the addition of sterilized distilled water to the silage samples (1:1 w/v); the juice was then filtered using sterilized double cheeseclothes and then placed into a 15 mL sterilized corning tube.

### Silage quality evaluation

Raw material and silage samples were analyzed using the proximate standard procedures of AOAC (1997), and the fiber fraction content protocol by Van Soest et al. (1991). The CP and ether extract content (EE) were analyzed using FOSS equipment (Kjeltec 8400 analyzer unit and Soxtec 2050, Hoganas, Sweden). Neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), hemicellulose, and cellulose were analyzed using Fibertec apparatus (FOSS Fibertec 2010, Hoganas, Sweden). Other parameters were also tested, including temperature, pH using Cyberscan pH310 Eutech, concentration of lactic acid by the titration method, NH<sub>3</sub>-N by the Conway method, WSC by a colorimetric method (Dubois et al., 1956), VFA using GC-FID (Bruker Scion 436, Fremont, CA, USA) and TP and TT by the Folin-Ciocalteu method (Makkar, 2003). The LAB population was determined by total plate count using MRS agar (Cappucino and Sherman, 2001). Microbial diversity was determined by terminal restriction length polymorphism (T-RFLP) analysis (Sakamoto et al., 2003; 2006).

### Terminal restriction length polymorphism analysis

Microbial DNA from silage juice was extracted using a genomic DNA mini kit, which is based on the 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). DNA was then incubated at  $60^\circ\text{C}$  for 30 min. Specimen DNA was pooled from each treatment (five DNA samples in total).

DNA was amplified using primer 6FAM-27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The amplification of the polymerase chain reaction (PCR) reaction was performed as described previously (Sakamoto et al., 2006), with total volume of 50  $\mu\text{L}$  consisting of 5  $\mu\text{L}$  dissolved DNA ( $<1 \mu\text{g}$ ), 0.5  $\mu\text{L}$  of 1.25 U Takara Ex Taq (Takara Shuzo Co., Shiga, Japan), 5  $\mu\text{L}$  of 10 $\times$  Ex Taq buffer, 4  $\mu\text{L}$  of dNTP mixture (2.5 mM each) and 10 pmol of each primer. 16S rDNA was amplified using a Biometra Thermocycler TGradient using the following program:  $95^\circ\text{C}$  for 3 min, followed by 30 cycles consisting of  $95^\circ\text{C}$  for 30 s,  $50^\circ\text{C}$  for 30 s and  $72^\circ\text{C}$  for 1.5 min, with a final extension at  $72^\circ\text{C}$  for 10 min.

Amplified DNA was verified by electrophoresis of the PCR product (5  $\mu$ L) using 1.5% agarose. PCR products were purified using an ultraclean PCR kit (Mo Bio Laboratories, Inc.) and then stored at  $-20^{\circ}\text{C}$  until analysis.

The T-RFLP was analyzed based on the method by Sakamoto et al. (2003), with some modifications. The purified PCR product (2  $\mu$ L) was digested with three restriction enzymes consisting of 20 U of *AluI*, *MspI*, and *RsaI* (Takara Shuzo, Japan) in a total volume of 10  $\mu$ L at  $37^{\circ}\text{C}$  for 1 h. The restriction digest product (2  $\mu$ L) was mixed with 8  $\mu$ L of Hi-Di Formamide and 1  $\mu$ L of standard Gene Scan 1200 LIZ (Applied Biosystems, Foster City, CA, USA). Each sample was denatured at  $95^{\circ}\text{C}$  for 2 min and then immediately placed on ice. The length of T-RF was determined using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). T-RFs were estimated by using local method peak scan version 2.0 (Applied Biosystems, USA). T-RFs with an area peak of less than 2% of the total area were excluded from the analysis (Sakamoto et al., 2003; 2006). DNA fragments were resolved to one base pair by manual alignment of the standard peaks from different electrophoretograms. Prediction of T-RFs was performed by using the MiCAIII microbial diversity database (Shyu et al., 2007). Phylogenetic relationships were determined from predicted T-RFs using the DDBJ and RDP II databases (Cole et al., 2009). Dendrogram analysis was based on the similarity coefficient for the objective interpretation of the difference of T-RF patterns using the unweighted pair-group method with arithmetic mean (UPGMA) (Blackwood et al., 2003). Diversity of microbial populations was determined based on the Shannon index method, as previously described by Magurran (2004).

### Statistical analysis

Data of fermentation characteristics were analyzed using analysis of variance with SPSS 16 for Microsoft Windows. Significant effects of treatments were further analyzed using the least significant differences Duncan's

**Table 1.** Chemical composition of raw materials

Composition	<i>P.</i>	<i>C.</i>	RAC
	<i>purpureum</i>	<i>calothyrsus</i>	
Dry matter (DM) (g/kg)	312.5	450.5	853.1
Organic matter (g/kg DM)	895.5	947.9	845.1
Crude protein (g/kg DM)	45.2	212.1	93.5
Ether extract (g/kg DM)	4.7	19.0	41.7
Neutral detergent fiber (g/kg DM)	746.2	551.8	382.8
Acid detergent fiber (g/kg DM)	510.1	488.8	268.0
Acid detergent lignin (g/kg DM)	382.5	215.7	140.6
Hemicellulose (g/kg DM)	236.2	63.0	114.8
Cellulose (g/kg DM)	73.5	299.8	100.5
Total phenols (g/kg DM)	36.0	165.4	NA
Total tannin (g/kg DM)	8.7	142.7	NA

RAC, ready available carbohydrate; NA, not available.

Test ( $p < 0.01$ ); microbial diversity data were analyzed descriptively.

## RESULTS

### Fermentation characteristics

Chemical and nutrient compositions of the raw materials and silage samples are shown in Table 1 and 2. Inclusion of different levels of *C. calothyrsus* significantly increased ( $p < 0.01$ ) the contents of dry matter (DM), organic matter, CP, and EE of the silages. The silages varied in DM content. DM of R0 was lower ( $p < 0.01$ ) than R2, R3, and R4. The content of CP in R0 was the lowest and significantly different ( $p < 0.01$ ) compared to the other treatments. Meanwhile, the content of the fibrous fractions of each silage varied among treatments. The NDF, ADF, ADL, and hemicellulose contents were consistently decreased with increasing levels of *C. calothyrsus* except for cellulose. The cellulose content of R0 silage was the lowest ( $p < 0.01$ )

**Table 2.** Nutrient composition and fiber fraction of silages

Composition	Treatment <sup>1</sup>				
	R0	R1	R2	R3	R4
Dry matter (DM) (g/kg)	311.9 <sup>a</sup>	335.5 <sup>ab</sup>	369.7 <sup>bc</sup>	407.1 <sup>c</sup>	465.4 <sup>d</sup>
Organic matter (g/kg DM)	875.0 <sup>a</sup>	897.0 <sup>b</sup>	909.4 <sup>bc</sup>	923.8 <sup>cd</sup>	934.0 <sup>d</sup>
Crude protein (g/kg DM)	56.0 <sup>a</sup>	105.7 <sup>b</sup>	142.5 <sup>c</sup>	172.6 <sup>d</sup>	202.2 <sup>e</sup>
Ether extract (g/kg DM)	16.1 <sup>a</sup>	13.0 <sup>a</sup>	27.9 <sup>b</sup>	31.8 <sup>bc</sup>	34.5 <sup>c</sup>
Neutral detergent fiber (g/kg DM)	665.8 <sup>c</sup>	597.0 <sup>b</sup>	559.6 <sup>a</sup>	544.1 <sup>a</sup>	538.4 <sup>a</sup>
Acid detergent fiber (g/kg DM)	492.1 <sup>b</sup>	452.7 <sup>a</sup>	446.1 <sup>a</sup>	447.3 <sup>a</sup>	448.8 <sup>a</sup>
Acid detergent lignin (g/kg DM)	343.6 <sup>d</sup>	253.8 <sup>c</sup>	261.5 <sup>c</sup>	174.6 <sup>b</sup>	134.1 <sup>a</sup>
Hemicellulose (g/kg DM)	173.7 <sup>d</sup>	144.4 <sup>c</sup>	113.5 <sup>b</sup>	96.8 <sup>ab</sup>	89.6 <sup>a</sup>
Cellulose (g/kg DM)	87.9 <sup>a</sup>	157.0 <sup>b</sup>	150.5 <sup>b</sup>	254.6 <sup>c</sup>	307.0 <sup>d</sup>

<sup>1</sup> R0, *P. purpureum* 100%; R1, *P. purpureum* 75%+*C. calothyrsus* 25%; R2, *P. purpureum* 50%+*C. calothyrsus* 50%; R3, *P. purpureum* 25%+*C. calothyrsus* 75%; and R4, *C. calothyrsus* 100%.

<sup>a-e</sup> Values with different superscripts are significantly different from each other ( $p < 0.01$ ).

**Table 3.** Fermentation characteristics of silage

Characteristics	Treatment <sup>1</sup>				
	R0	R1	R2	R3	R4
Temperature (°C)	28.53 <sup>a</sup>	28.87 <sup>a</sup>	29.03 <sup>ab</sup>	29.50 <sup>bc</sup>	29.90 <sup>c</sup>
pH	3.52 <sup>a</sup>	3.51 <sup>a</sup>	3.65 <sup>ab</sup>	3.84 <sup>bc</sup>	4.00 <sup>c</sup>
LAB (log cfu/g)	5.40 <sup>a</sup>	6.49 <sup>bc</sup>	5.93 <sup>ab</sup>	6.74 <sup>bc</sup>	7.28 <sup>c</sup>
NH <sub>3</sub> -N (mM/g DM)	13.93 <sup>a</sup>	21.68 <sup>a</sup>	34.09 <sup>b</sup>	42.21 <sup>b</sup>	43.56 <sup>b</sup>
WSC (g/kg DM)	24.09	21.60	17.06	17.40	17.99
Lactic acid (g/kg DM)	32.8 <sup>b</sup>	29.8 <sup>ab</sup>	28.7 <sup>ab</sup>	22.9 <sup>a</sup>	20.4 <sup>a</sup>
Acetic acid (g/kg DM)	6.26	5.31	5.68	5.38	5.85
Propionic acid (g/kg DM)	0.23	0.13	0.58	0.95	0.15
Butyric acid (g/kg DM)	0.77	0.71	0.66	0.60	0.38
Total phenols (g/kg DM)	45.3 <sup>a</sup>	75.0 <sup>b</sup>	71.1 <sup>ab</sup>	87.2 <sup>b</sup>	94.1 <sup>b</sup>
Total tannin (g/kg DM)	14.0 <sup>a</sup>	39.2 <sup>b</sup>	48.3 <sup>bc</sup>	61.1 <sup>c</sup>	69.2 <sup>c</sup>

DM, dry matter; LAB, lactic acid bacteria; WSC, Water soluble carbohydrates.

<sup>1</sup> R0, *P. purpureum* 100%; R1, *P. purpureum* 75%+*C. calothyrsus* 25%; R2, *P. purpureum* 50%+*C. calothyrsus* 50%; R3, *P. purpureum* 25%+*C. calothyrsus* 75%; and R4, *C. calothyrsus* 100%.

<sup>a-c</sup> Values with different superscripts are significantly different from each other (p<0.01).

compared to all of the other treatments. The effect of the inclusion of *C. calothyrsus* in silage on fermentation characteristics is presented in Table 3. The temperature, pH, LAB population, NH<sub>3</sub>-N, TP, and TT all increased (p<0.01) significantly as the level of *C. calothyrsus* increased. Lactic acid concentration did not increase; silage with higher levels of *C. calothyrsus* had reduced lactic acid concentration compared to those containing lower levels of *C. calothyrsus*. The increase in the level of *C. calothyrsus*

**Table 4.** Microbial diversity index in silage

Index value	Treatment <sup>1</sup>		
	R0	R2	R3
Richness (S)	7(±3)	3(±0.58)	3(±1.15)
Shannon (H')	2.07(±0.93)	0.88(±0.21)	0.65(±0.51)

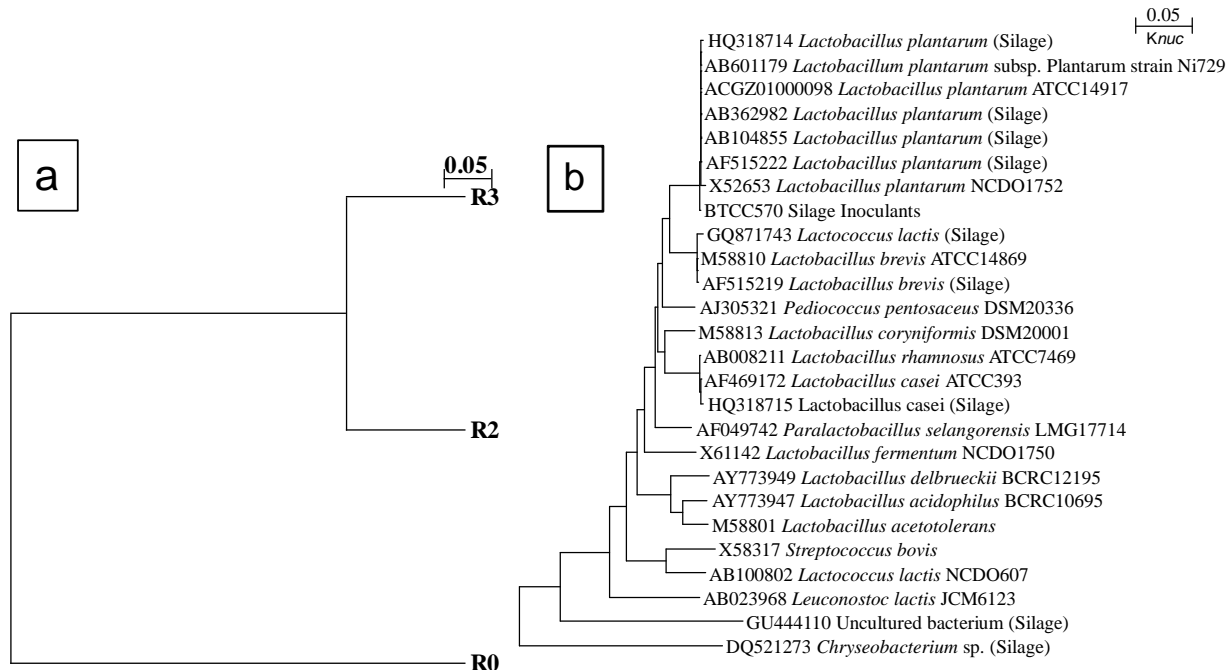
<sup>1</sup> R0, *P. purpureum* 100%; R2, *P. purpureum* 50%+*C. calothyrsus* 50%; and R3, *P. purpureum* 25%+*C. calothyrsus* 75%.

had tendency to reduce the content of WSC and butyric acid on a DM basis.

### Microbial diversity in silage

A total of five samples were subjected to PCR with 16S rDNA universal primers but 16S rDNA could not be amplified from two samples (R1 and R4). The PCR products obtained from the two samples were not suitable for T-RFLP analysis due to weak amplification. Consequently, three samples were subjected to T-RFLP analysis. The microbial diversity calculated by the Shannon index varied among treatments and a decrease was observed in the bacterial population diversity as levels of *C. calothyrsus* increased (Table 4). The increase of the diversity index in silage indicated that each sample contained diverse populations of microorganisms. Sample R0 showed the highest value of T-RF richness and Shannon index compared to the other treatments, while R3 had the lowest Shannon index.

The close similarity of microorganisms in silage was confirmed by the dendrogram (Figure 1a and 1b). It was constructed by combining three T-RFLP patterns with three



**Figure 1.** Close similarity of silage microbial (a) and phylogenetic relationship (b). R0, *P. purpureum* 100%; R2, *P. purpureum* 50%+*C. calothyrsus* 50%; and R3 *P. purpureum* 25%+*C. calothyrsus* 75%.

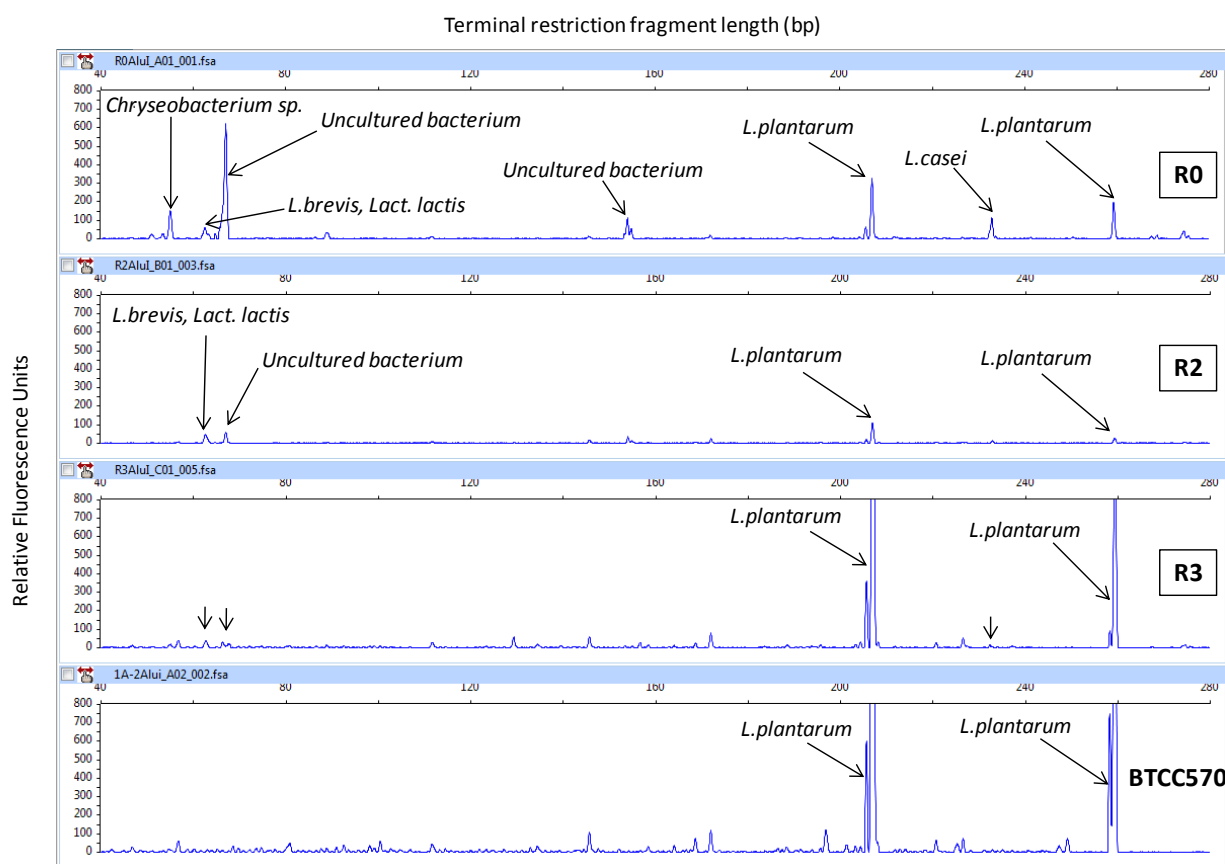
different restriction enzymes by UPGMA methods (Figure 1a). Samples R2 and R3 were shown to have similar microorganisms. The phylogenetic tree was constructed by alignment of the T-RF database from DDBJ and RDP II type strains (Figure 1b). It described T-RFs of each silage treatment which showed close similarity with the other database. T-RFs from silage treatments were predicted to high similarity compared with the MiCAIII database. Figure 2 shows the changes in microbial diversity of silage with different levels of *C. calothyrsus*. The microbial community analysis clarified by T-RFLP indicated that there was a population of four LAB phylotypes, *Chryseobacterium* sp., and uncultured bacteria (Figure 2). Microorganisms in the silage were dominated by LAB. The LAB population had close similarity with *L. plantarum*, *L. casei*, *L. brevis*, and *Lactococcus lactis*.

## DISCUSSION

The combination of grasses and legumes in ruminant feed is very effective for a highly nutritional diet (Sturm et al., 2007). The purpose of the addition of legumes to silage is to supply N-protein for microbial protein synthesis,

reduce protein degradation in the rumen, and increase amino acid absorption in the intestinal tract. Gómez-Vázquez et al. (2011) and Wanapat et al. (2014) expressed that silages can be used for alternative feed supplement to improve ruminant performance. The feed containing the highly nutritive grass-legume combination generally needs to be preserved for a long time. A pH of less than 4 can be used to preserve feed for a long period, which reflects the adequacy of fermentation to restrict the growth of undesirable microorganisms (Arriola et al., 2011). McDonald et al. (1991) described that the characteristics of good silage should have pH values of 4.20 or less,  $\text{NH}_3\text{-N}$  contents less than 100 g/kg DM, and high lactic acid contents. In this study, all silages were well-preserved, with high lactic acid contents and low pH values (Table 3). During the ensilage process, LAB produced lactic acid to lower the pH and inhibit the growth of undesirable microorganisms, and produced good quality silage. The silage from sample R2 had adequate silage nutritional values based on the standard requirements of ruminant feed.

There was a unique relationship between silage fermentation characteristics and microbial diversity; pH, LAB, and TT increased, but lactic acid decreased with



**Figure 2.** T-RFLP patterns of silage and *L. plantarum* BTCC570 after digestion with restriction enzyme *AluI*. R0, *P. purpureum* 100%; R2, *P. purpureum* 50%+*C. calothyrsus* 50%; and R3, *P. purpureum* 25%+*C. calothyrsus* 75%. T-RFLP, terminal restriction length polymorphism.

increasing levels of the legume (Table 3). The increasing LAB population generally increases lactic acid concentration, but in this study the opposite was observed. This finding suggests that legumes used had low WSC and that TT inhibited lactic acid production of LAB. Norton (1994) and McDonald et al. (1991) stated that legumes are generally high in protein content and buffering capacity, but low in WSC content. The high level of tannins used can also reduce the activities of silage bacteria (Salawu et al., 1999). Both TP and TT contents contributed to the decrease in lactic acid production. These results agree with those from Deaville et al. (2010) who found that tannin suppressed the production of lactic acid during ensilage. In this case, lower TT in sample R0 produced higher lactic acid compared to the other treatments (Table 3). Sonja et al. (2012) reported that low pH and anaerobic conditions reduced TT content. The TT content of silage decreased 51.5% in R4 after 30 days incubation. A high concentration of polyphenolics such as TT in the feed can inhibit digestibility, absorption and reduce CH<sub>4</sub> production. Enteric CH<sub>4</sub> production in rumen fermentation has been intensively studied, and the beneficial effect of tannin to reduce CH<sub>4</sub> emission and gas production has also been reported by several authors (Castro-Montoya et al., 2011; Jayanegara et al., 2011a, b; Bodas et al., 2012; Kondo et al., 2014). Jayanegara et al. (2011b) reported that TT can be used at a concentration of less than 100 g/kg DM, which is equivalent to 10% DM in the feed formulation for cattle.

Generally, the first stage of ensiling is competition among the epiphytic microorganisms to use WSC to produce fermentation products. Undesirable microorganisms such as enterobacteria, clostridia, and fungi also grow in the silage, causing nutritional losses. Li and Nishino (2013) reported that several non-LAB bacteria such as *Pantoea agglomerans* and *Enterobacter* sp. were found in the pre-ensiled *Italian ryegrass*. *Chryseobacterium* sp., and uncultured bacteria were detected in R0 tendency to produce high contents of acetic acid and butyric acid (Figure 2, Table 3). The methods for inhibiting the growth of undesirable microorganisms include the addition of LAB inoculants, substitution with other materials containing high DM, such as legume, or reducing the moisture content of the crops (McDonald et al., 1991; Arriola et al., 2011). The purpose of LAB inoculants is to compete with the epiphytic microorganisms by increasing lactic acid production to inhibit undesirable microorganisms. The changes in microbial population in the first stage of the ensiling process are critical to the success of the fermentation process (Mulrooney and Kung, 2008).

In this research, silage was also made by using LAB inoculants to increase the LAB population, in order to stimulate lactic acid production, accelerate the decrease in pH, and increase silage quality. Microbial diversity in the

silage can determine the silage quality. The increasing level of *C. calothyrsus* in the silage decreased the microbial diversity index. The LAB has the potential to increase the production of lactic acid because it is homofermentative (McDonald et al., 1991; Lynch et al., 2012). This finding suggests that the *L. plantarum* population was sufficiently established during ensiling process and able to utilize the high concentration of NH<sub>3</sub>-N. The NH<sub>3</sub>-N in the silage was used as a source of N for microbial protein synthesis, for microbial growth (McDonald et al., 1991; Norton, 1994). The growth of the *L. plantarum* population was resistant to tannin but its activity decreased with high levels of *C. calothyrsus*. The resistance mechanism may be correlated to the production of lactic acid. Patra et al. (2012) reported that heme-containing enzyme from *Lactobacillus* is not present and produce exo-polysaccharides that form the protective layer around the cell. This mechanism potentially inhibited the lactic acid production. Other known resistance mechanisms of *L. plantarum* include the ability to degrade phenolic compounds (Landete et al., 2008; Rodríguez et al., 2009) such as tannin, by the action of novel tannase (*tanLpI*) (Iwamoto et al., 2008) and gallate decarboxylase enzymes (*lpdB* and *lpdC*) (Jiménez et al., 2013). In the present study, the silage contained four homofermentative type LAB, of which the most dominant was *L. plantarum* (Figure 2). Lynch et al. (2012) reported that the *L. plantarum* population in silage without or with the addition of the inoculants was more dominant than the population of *L. buchneri*. The population of *L. plantarum* was shown to be dominant in wilted guinea grass silage stored for 28 days, followed by *L. brevis*, *L. lactis*, *Enterobacter* sp., and *L. garvieae* (Li and Nishino, 2013).

## CONCLUSION

Increasing levels of *C. calothyrsus* had a significant effect on the fermentation characteristics, such as the nutrient and fiber composition, and reduced the microbial diversity index of silage. The quality of tropical silage containing *C. calothyrsus* was shown to be good, had excellent fermentation characteristics and the microbial communities were dominated by *L. plantarum*.

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### CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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