Fermentation Kinetics (*In Vitro*) of *Leucaena leucocephala, Gliricidia sepium* and *Calliandra callothyrsus* Leaves (3) the Pattern of Gas Production, Organic Matter Degradation, pH, NH_3 and VFA Concentration; Estimated CH_4 and Microbial Biomass Production

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ABSTRAK

WIDIAWATI, Y., M. WINUGROHO, E. TELENI dan A. THALIB. 2007. Kinetik fermentasi (*in vitro*) daun *Lucaena leucocephala, Gliricidia sepium* dan *Calliandra callothyrsus* (3) pola produksi gas, degradasi bahan organik, nilai pH, konsentrasi NH_3 dan VFA; perkiraan produksi gas CH_4 dan biomasa mikroba. *JITV* 12(3): 202-211.

Kecernaan pakan kaya protein biasanya terhambat oleh adanya proses lignifikasi pada dinding sel tanaman dan hadirnya senyawa sekunder seperti tannin yang dimiliki oleh hampir semua jenis tanaman leguminosa. Kondisi ini akan berpengaruh terhadap kecernaan bahan organik dan pola produk akhir fermentasi pakan oleh mikroba rumen (VFA, NH_3, gas total dan CH_4). Teknik *in vitro* digunakan untuk mengetahui kecernaan bahan organik dan produk akhir fermentasi mikroba rumen pada tiga pohon leguminosa yaitu *Leucaena leucocephala, Gliricidia sepium* and *Calliandra callothyrsus.* Hasil penelitian menunjukkan bahwa Gliricidia mempunyai jumlah tertinggi pada bahan organik tercerna (505 mg), produksi sel mikroba rumen dan NH_3 (2676 g dan 1,91 mmol/100 mL) diikuti oleh Leucaena (423 mg; 2656 g; 1,61 mmol/100 mL) dan Kalliandra (340 mg; 2644 g; 1,61 mmol/100mL), tetapi Gliricidia menghasilkan gas total dan CH_4 terendah (0,192 mL and 0,07 mole per mg bahan organik tercerna) diikuti oleh Leucaena (0,249 mL and 0,097 mole per mg bahan organik tercerna) dan Kalliandra (0,196 mL and 0,126 mole per mg bahan organik tercerna). Disimpulkan bahwa Gliricidia lebih efisien menggunakan energi pakan untuk meningkatkan produktivitas ternak dibandingkan dengan Leucaena dan Kalliandra.

Kata Kunci: Pohon Leguminosa, Bahan Organik, NH3, VFA, CH_4, Sel Mikroba

ABSTRACT

WIDIAWATI, Y., M. WINUGROHO, E. TELENI and A. THALIB. 2007. Fermentation kinetics (*in vitro*) of *Leucaena leucocephala, Gliricidia sepium* and *Calliandra callothyrsus* leaves (3) the pattern of gas production, organic matter degradation, pH, NH_3 and VFA concentration; estimated CH_4 and microbial biomass production. *JITV* 12(3): 202-211.

In high protein feeds, plant proteins may be shielded from degradation in the rumen by lignification of the cell wall or because of the present of plant secondary compounds e.g. tannin, which is present in most of the leguminous trees/shrubs. Thus it might have an effect on organic matter degradation and the pattern of other end product of rumen microbial fermentation (VFA, NH_3, microbial cell, CH_4 and total gas). The *in vitro* method was used to determine the organic matter degradation and the pattern of rumen fermentation end product of high protein feeds, namely *Leucaena leucocephala, Gliricida sepium* and *Calliandra calothyrsus*. The results shows that Gliricidia has the highest amount of OM degraded (505 mg); microbial cell and NH_3 produced (2676 g and 1.91 mmol/100 mL) than followed by Leucaena (423 mg; 2656 g; 1.61 mmol/100 mL) and Calliandra (340 mg; 2644 g; 1.61 mmol/100mL), but Gliricidia produced the lowest amount of gas total and CH_4 (0.192 mL and 0.07 mole per mg OM deraded) then Leucaena (0.249 mL and 0.097 mole per mg OM deraded) and Callliandra (0.196 mL and 0.126 mole per mg OM deraded). In conclusion, Gliricidia is more efficient in term of using the dietary energy for the animals' production compared to the Leucaena and Calliandra.

Key Words: Legumes Tree, Organic Matter, NH_3, VFA, CH_4, Microbial Cell

INTRODUCTION

The differences on organic matter degradation and the pattern of rumen fermentation end product between two types of feeds, namely grass and shrub legume leaves (Leucaena) have been described in two previous papers (WIDIAWATI and THALIB, 2007a,b). Secondary compound, found in most of shrub legumes leaves, has an effect on protein and organic matter degradation, thus would influence the pattern of end product of rumen fermentation. Three shrub legumes, *Leucaena leucocephala, Gliricidia sepium* and *Calliandra calothyrsus* contain different type of secondary compound. The three shrub legumes are widely used as

feed supplement for ruminant animal both in Indonesia and in the world.

The extent of crude protein degraded in the rumen would depend on the forms of the protein and on the presence of secondary compounds. There are two general forms of protein present in a plant material, namely the water-soluble and non water-soluble proteins. Water-soluble protein is rapidly degraded while non water-soluble protein is more slowly broken down in the rumen (POPPI and NORTON, 1995). Soluble protein content varies widely among forages. High content of soluble protein (20-60%) may be found in high-protein protein such as leucaena, Gliricidia and Calliandra (PRESTON and LENG, 1987).

Secondary compounds are present in most of the legume leaves. The compounds that are found in the shrub legumes Leucaena, Gliricidia and Calliandra are mimosine, coumarine and tannin, respectively. Tannin also presents in Leucaena and Gliricida, but in smaller amounts (3-4%) compared to the amount that is present in Calliandra being 19,4% (JAKCSON *et al.*, 1992; MULLEN, 1996; SHELTON *et al.*, 1996). It was reported that secondary tannin had negative effect on protein degradation by rumen microbes (NORTON, 1994; MULLEN, 1996). Since protein content of the three shrub legumes is about one third of total organic matter, therefore the amount of organic matter degraded would be influenced by the amount of protein degraded. Therefore, the paper presented will described the differences in organic matter degradation and rumen fermentation end product of the three shrub legumes.

MATERIALS AND METHODS

Experimental design

Fermentation kinetics (*in vitro*) of three shrub legumes, namely Leucaena, Gliricidia and Calliandra were examined using a Completely Randomized Design (DANIEL, 1991). The *in vitro* method developed by THEODOROU and BROOKS (1990) for the study of fermentation kinetics was employed in the current experiment.

Feed samples, microbial inoculum and incubation medium

Feed samples of Leucaena, Gliricidia and Calliandra, rumen digesta and the medium for incubation were prepared as described in WIDIAWATI and THALIB (2007a).

Procedure

The procedure of medium preparation before microbial inoculums inoculated into the bottle incubation was followed the procedure as described in WIDIAWATI and THALIB (2007a).

Sampling and Laboratory analysis

Organic matter degradation and gas production

The feed OM degradation and gas production were determined by using the techniques described in WIDIAWATI and THALIB (2007a).

pH, NH_3 and VFA production

The value of pH, concentration of NH_3 and VFA of the medium in each inoculated bottle was determined as described in WIDIAWATI and THALIB (2007a).

Methane production

The equations to predict the amount of methane and microbial biomass produced during fermentation of the feeds in the culture bottles are similar to the equations described in previous paper (WIDIAWATI and THALIB, 2007b).

Statistical Analysis

All raw data were tabulated using Microsoft® Excel 2000 for Windows 2000 (Microsoft Corporation, USA) and analysed using SPSS Version 7.0 for Windows 95 (SPSS Inc, USA). Graphs were produced using Microsoft® Excel 2000 for Windows 2000. The data were analysed using one-way ANOVA (DANIEL, 1991) for Completely Randomized Design. Where significant effects from treatments were observed, differences among mean values were examined using Tukey's test (STEEL and TORRIE, 1980)

RESULTS

The three shrub legumes contain similar amount of protein (21-23%), cell wall and cell content as showed by percentage of acid detergent fibre (ADF) (28-31%) and neutral detergent fibre (NDF) (48–51%), respectively.

Organic matter degradation

Data on water-soluble, water-insoluble but degradable, total water-soluble plus water-insoluble but degradable fractions, and the rate of OM degradation the three shrub legumes is presented in Table 1.

The proportions of water-soluble fractions were not affected by type of legumes, however water-insoluble but degradable fraction was affected by the type of

legumes. The three legumes had similar water-soluble contents. The Gliricidia had the highest content of water-insoluble but degradable fraction and total water-soluble and water-insoluble but degradable fraction ($P<0.01$) compared to other shrub legume. Corresponding values to these were lowest for Calliandra. The lowest rate of OM degradation during the 0-24 h incubation period was for Calliandra ($P<0.01$) followed by rates for Leucaena and Gliricidia, in ascending order. However, the Calliandra had the highest rate of OM degradation during the 24-48 h incubation period followed by rates for Gliricidia and Leucaena in descending order ($P<0.01$).

The patterns of feed OM degradation in the culture bottles during 48 h of incubation are presented in Figure 1. The OM of Gliricidia and Leucaena were degraded at steady rates, both of which were higher than that of Calliandra.

By using the OM degradation curve of a feed, it was possible to determine the water-soluble fraction of the feed. The water-soluble fractions of Gliricidia and leucaena were each higher than that of the Calliandra. These results would have contributed to the higher rate of OM degradation observed in these two legumes compared with the Calliandra. Calliandra has a similar NDF content to those of Gliricidia and Leucaena but was degraded more slowly. It was suggested that the high tannin content of Calliandra might have been the main reason for its low rate of degradation in the rumen (MCSWEENEY *et al.*, 1999a).

Table 1. Means of water-soluble (a), water-insoluble but degradable (*b*) and *a* + *b* fractions at 24 h and 48 h of incubation and the degradation rate (0 - 24 h and 24 - 48 h of incubation) of organic matter (OM) from Leucaena, Gliricidia and Calliandra

Variables	Feed samples				
	Leucaena	*Gliricidia*	*Calliandra*	± SE	P
a. Fraction (mg)	26	27	25	2.9	0.05
b Fraction (mg)					
24 h	375[b]	449[a]	240[c]	22	0.000
48 h	426[b]	516[a]	320[c]	156	0.000
a + *b* Fraction (mg)					
24 h	401[b]	476[a]	265[c]	29	0.001
48 h	452[b]	543[a]	345[c]	20	0.000
Rate of OM degradation (mg/h)					
0 - 24 h	16.7[b]	19.8[a]	11[c]	0.95	0.000
24 - 48 h	2.1[b]	2.8[b]	3.3[a]	0.10	0.003

Within rows, means with different superscripts differ significantly ($P<0.05$)

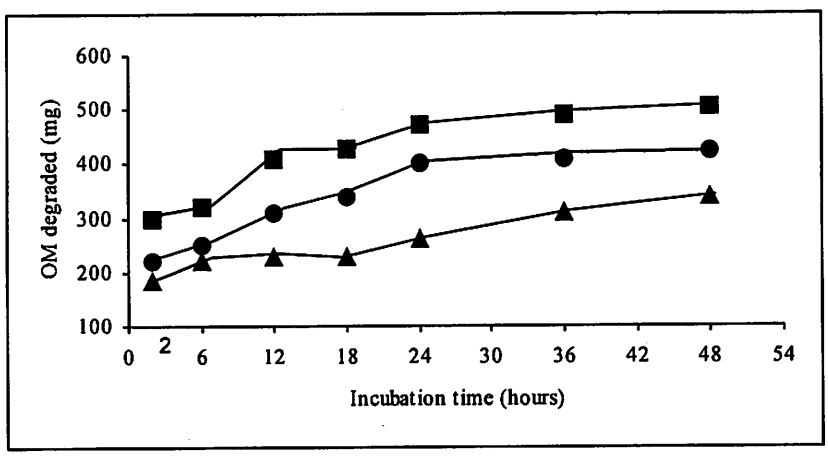

Figure 1. Means of organic matter (OM) of Leucaena (●), Gliricidia (■) and Calliandra (▲) degraded during 48 hours of incubation

The tannin content of the feeds examined was not determined in the current experiment. Published values, however, show that Calliandra would contain relatively high amounts of tannin (e.g., 19.4% in DM) while lower amounts (e.g., 3.4% and 4%) would be found in Gliricidia and Leucaena, respectively (JACKSON *et al.*, 1992; MULLEN, 1996; SHELTON *et al.*, 1996). The high tannin present in the Calliandra probably protects the legume from degradation by forming complexes with the carbohydrates and proteins (MCSWEENEY *et al.*, 2001).

The results showed that the presence of coumarin and mimosine in Gliricidia and Leucaena (AHN *et al.*, 1989; CHADHOKAR, 1982) had no effect on organic matter degradation by rumen microbes. It was likely that low content of tannin in these two shrub legumes (3.3 – 4.0%) (AHN *et al.*, 1989; CHADHOKAR, 1982) not influenced the amount of OM degradation in the rumen.

Gas production

Data on the total volume of gas produced and the rate at which the gas was produced from fermentation of the different legume are presented in Table 2.

The total volume of gas produced in the culture bottles were significantly affected by the type of legume. The Calliandra had the lowest total volume of gas produced ($P<0.01$), while mean values were similar for Gliricidia and Leucaena. There were differences in the rates of gas production among the feeds during the first and second 24 h of incubation. In the first 24 h, Gliricidia and Leucaena had higher rates of gas production than did Calliandra ($P<0.01$). In the second 24 h of incubation, however, the rates of gas production from the three legumes were similar (see Table 2).

The patterns of volume of gas production per mg of feed OM are presented in Figure 2. Leucaena produced the highest volume of gas per mg OM degraded. Volume of gas produced per mg of OM was higher for Gliricidia than for Calliandra between 0 - 18 h of incubation, but the situation was reversed during 18 h - 48 h of incubation.

The total volume of gas produced during 48 h of incubation of feed samples was similar for Gliricidia and Leucaena and higher (33 and 38%, respectively) than that for Calliandra. When the values for gas produced were expressed per mg of OM degraded, the three shrub legumes produced less gas (13-44%) than that the grass (THALIB and WIDIAWATI, 2007a). This reflects the probable lower energetic efficiency associated with the conversion of the grass OM to absorbable nutrients.

Table 2. Means of total gas volume produced, and rate of gas production during fermentation (0 h - 24 h and 24 h - 48 h) of *Leucaena, Gliricidia* and *Calliandra*

Variables	*Leucaena*	*Gliricidia*	*Calliandra*	+ SE	P
Total gas volume produced (mL)	144[a]	138[a]	104[b]	8.09	0.010
Gas production rate (mg/h)					
0 - 24 h	4.7[a]	4.4[ab]	2.7[c]	0.30	0.001
24 - 48 h	1.3	1.4	1.6	0.37	0.055

Within rows, means with different superscripts differ significantly ($P<0.05$)

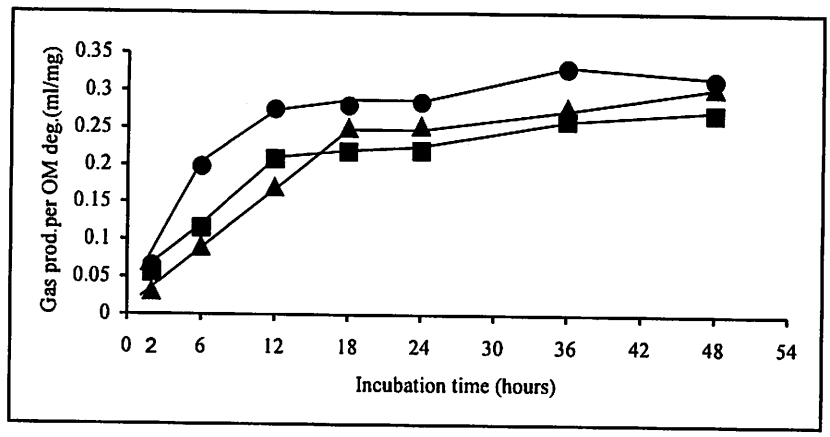

Figure 2. Means of volume of gas produced per mg of organic matter (OM) of *Leucaena* (●), *Gliricidia* (■) *and Calliandra* (▲) degraded during the 48 hours of incubation

The lower volume of gas produced in Calliandra fermentation would appear to reflect simply the lower degradability of the particular legume. The question of interest, however, is whether the lower gas production from the Calliandra was due to a general lower OM availability or a specific inhibition of cellulose digestion by tannin (BAE *et al.*, 1993) and/or an inhibition of gas production during the fermentation (NSAHLAI *et al.*, 1994).

The data showed that Calliandra contained an amount of water-soluble fraction similar to amounts in Gliricidia and Leucaena, but the rate of gas production from the Calliandra was significantly lower than rates recorded for Gliricidia and Leucaena. It therefore would appear that the availability of OM in Calliandra might not have been the major reason for the lower gas production rate from the legume. Rather, it might have been a specific inhibitory effect of tannin, possibly, on cellulose digestion and/or gas production that were reflected in the lower rate of gas production.

Volatile fatty acids production

The patterns of means of VFA concentration and of VFA concentration per mg of feed OM degraded are presented in Figures 3 and 4, respectively.

The concentrations of VFA were similar at 2 h of incubation, being 3.2 – 3.3 mM. The rapid increase in VFA concentration in all the feeds in the first 2 h of incubation is indicative of the rapid rate of fermentation during this period probably due to the presence of the water-soluble fractions of these feeds. Among the three legumes, Gliricidia produced more VFA then followed

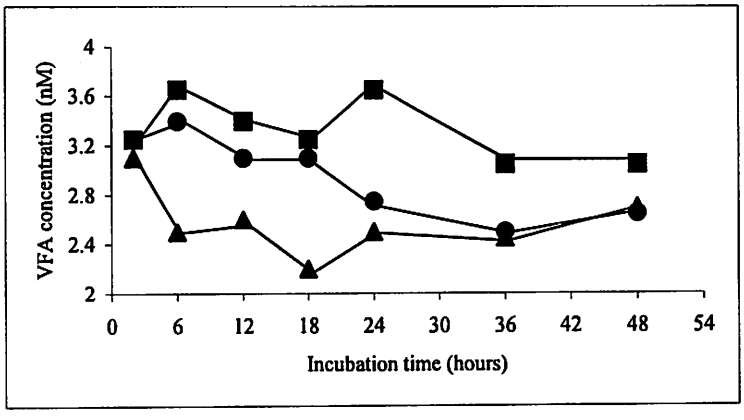

Figure 3. Means of volatile fatty acids (VFA) concentration of Leucaena (•), Gliricidia (■) and Calliandra (▲) during the 48 hours of incubation

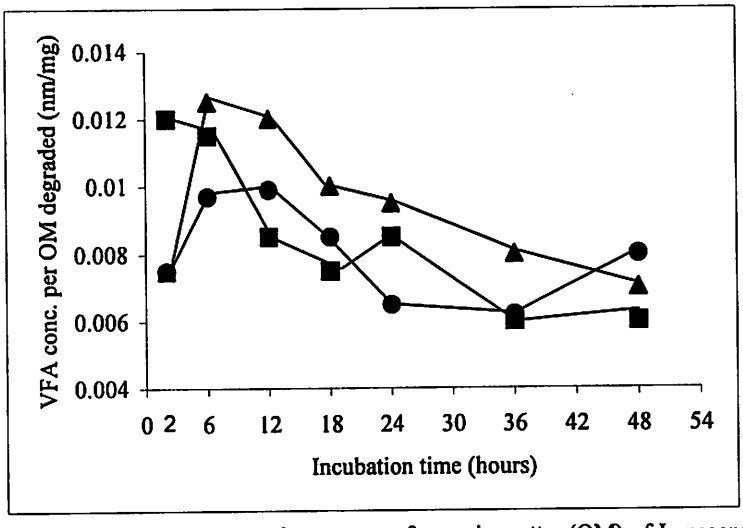

Figure 4. Means of volatile fatty acid (VFA) concentration per mg of organic matter (OM) of Leucaena (•), Gliricidia (■) and Calliandra (▲) degraded during the 48 hours of incubation

by Leucaena and Calliandra, but these differences were not different significantly.

The concentrations of VFA peaked at 6 h, 2 h and 2 h of incubation for Gliricidia, Leucaena and Calliandra, respectively. Since the VFA concentrations were not recorded hourly in the current experiment, the times for peak VFA concentration to be reached in each feed may lie between the peak times given above and the next recording times, i.e., 12 h, 6 h and 6 h. In *in vivo* studies (e.g., CHURCH, 1976), VFA concentrations in the rumen were observed to reach its peak at 4 h and 6 h after the animals received their ration namely alfalfa and wheaten hay, respectively

The value of VFA concentration per mg of OM degraded was similar throughout the incubation period for all the legumes. In general, the concentration of VFA per mg of OM degraded in the feeds examined peaked at incubation times between 2 h and 5 h.

The mean molar proportions of the three main VFA; acetate, propionate and butyrate, during 48 h of incubation are presented in Figures 5 (a, b and c). The patterns of mean molar proportions of the three VFA

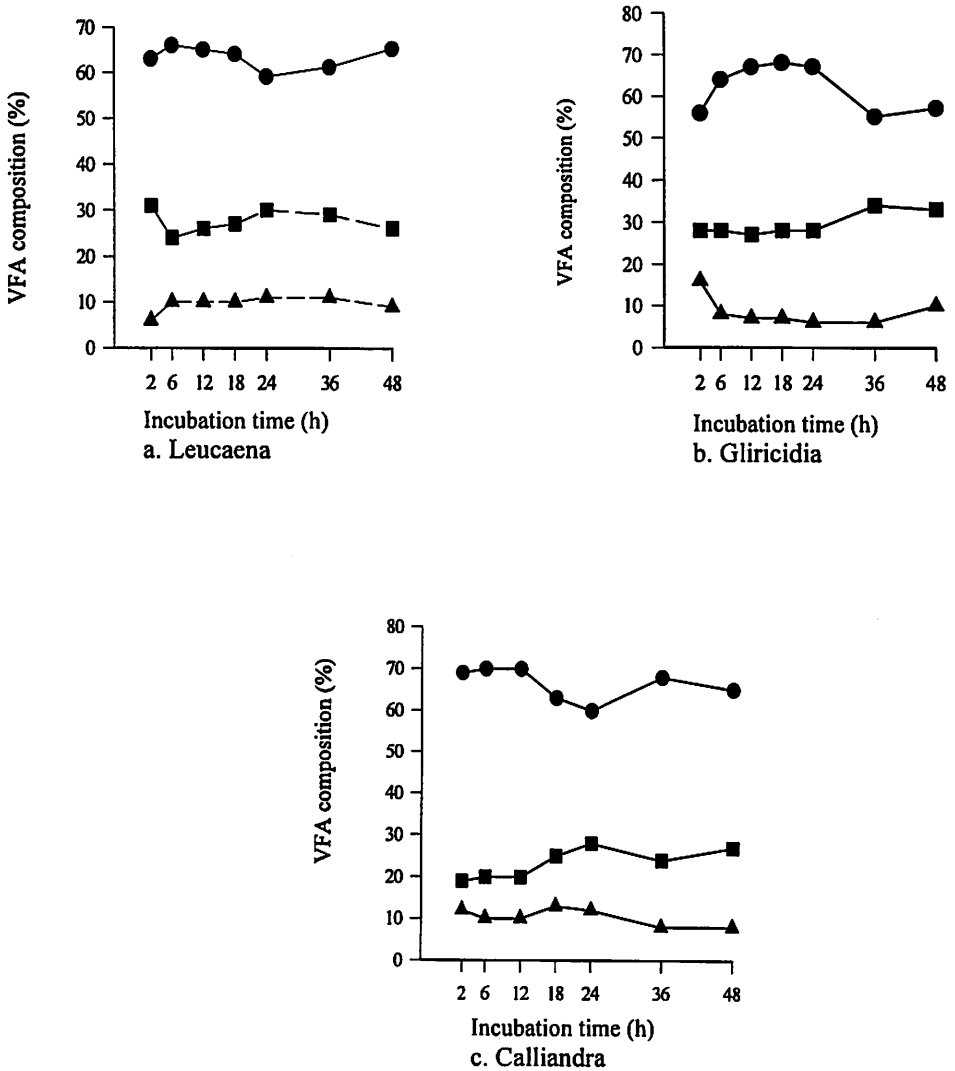

Figure 5. Mean molar proportion of volatile fatty acid (VFA) occurring as acetate (●), propionate (■), and butyrate (▲) from (a) Leucaena, (b) Gliricidia and (c) Calliandra, during the 48 hours of incubation

were similar generally for the legumes. The figures show an apparent inverse relationship between the molar proportions of acetate and propionate for all feeds. The Gliricidia and Leucaena had higher proportions of propionate throughout most of the incubation period (see Figures 5 a, b and c).

Differences in the composition of amino acid in the three legumes examined might contribute to differences in the proportions of VFA produced. Propionate mostly is produced from amino acids such as arginine, ornithine, proline, lysine and δ-amino valeric acid, while butyric acid is produced from valine, leucine, and isoleucine (HUNGATE, 1966). The three legumes examined contained high amount of those amino acids. The composition of essential amino acid of the three shrub legumes showed in Table 3.

Table 3. The essential amino acids and arginine composition in Leucaena, Gliricidia, and Calliandra used in the experiment

Essential amino acids (μg/g N)	Feeds		
	Leucaena	Gliricidia	Calliandra
Histidine	205	219	231
Isoleucine	536	563	564
Leucine	504	521	543
Lysine	535	472	614
Methionine	239	218	289
Phenylalanine	645	704	687
Threonine	549	593	590
Valine	637	646	685
Arginine	725	779	811

Source: WIDIAWATI (2002)

pH and NH_3

The means of pH values of the medium during the 48 h of incubation are presented in Table 4.

The feed had no significant effect on the pH of the medium in the culture bottles during the 48 h incubation period. The pH values were relatively steady during the incubation period and ranged from 6.6 to 7.3 for the feeds examined. The results show that the pH values of media in culture bottles were not significantly affected by the type of shrub legumes. The values were in the rumen pH range of 6.5 to 7 considered suitable (HUNGATE, 1966) for optimal activity and growth of the two major groups of bacteria, namely, the cellulolytic and proteolytic bacteria. There was a tendency for the pH values of the media containing Gliricidia to be higher than values observed for the other feeds. This is consistent with the observation that the legume was associated with a higher concentration of ammonia.

The NH_3 concentration values were affected significantly by the type of feeds. Although values were similar for all the feeds at 2 h and 6 h of incubation, these started to differ significantly from 12 h onwards of incubation. The values for Gliricidia were consistently above those of Leucaena and Calliandra. ($P<0.05$). The Leucaena and Calliandra had similar values for NH_3 concentration during the incubation period.

Table 4. Means of pH during fermentation of Leucaena, Gliricidia and Calliandra

Period	Treatment		
	Leucaena	Gliricidia	Calliandra
2 h	7.0	7.3	6.8
6 h	6.8	7.2	6.8
12 h	6.9	7.0	6.7
18 h	6.8	7.2	7.0
24 h	6.8	7.2	7.0
36 h	6.7	7.1	6.8
48 h	6.8	7.0	6.6

Overall, the values of NH_3 concentrations for all the feeds were above the minimum concentration (50 mg/L or 0.3 mmole/100mL) required for rumen microbial activities (SATTER and SLYTER, 1974). This might have been due, to a certain extent, to the fact that there was no loss of NH_3 from the fermentation system during the incubation period.

Observations from some studies (MULLEN, 1996) suggested that tannin-protein complexes that might form in Calliandra would protect the protein from microbial degradation. In the current experiment, some ammonia was released when Calliandra was fermented, indicating protein degradation in the legume. This is in agreement with the suggestion by MCSWEENEY *et al.* (1999b) that not all protein in Calliandra form complexes with tannin. Indeed they stated that there is a substantial amount of protein that would be readily available for microbial fermentation. What is not clear, however, is the percentage of protein in Calliandra, which would be free from tannin.

Methane production

Fermentation of feed yields a higher rate of production of acetate would be associated with a higher rate of production of CO_2; normally a major hydrogen

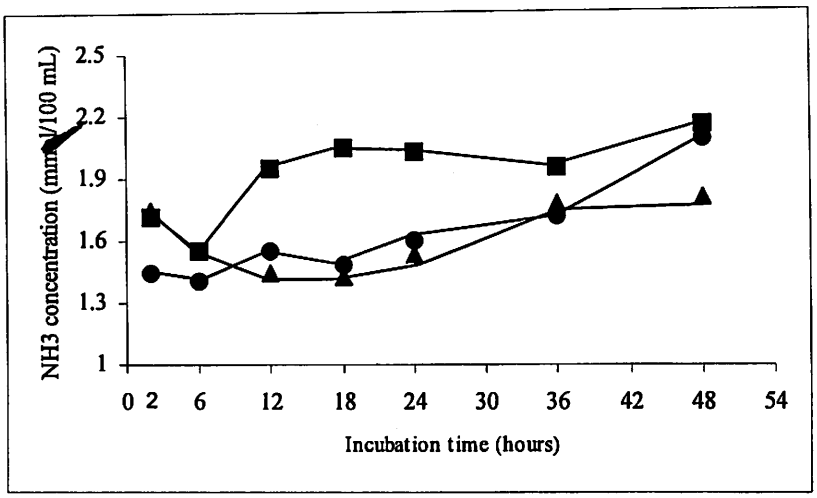

Figure 6. Mean NH_3 concentration of the medium when Leucaena (●), Gliricidia (■) and Calliandra (▲) were degraded during the 48 hours of incubation

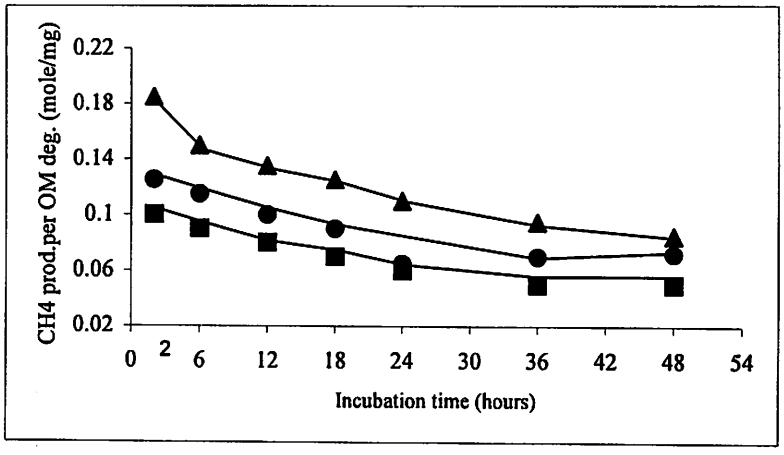

Figure 7. Estimation of methane (CH_4) produced, which was obtained from the concentration of acetate, propionate and butyrate when Leucaena (●), Giricidia (■) and Calliandra (▲) were degraded during the 48 hours of incubation

sink in the rumen, resulting in CH_4 production. The net hydrogen produced that would have been disposed of with CO_2 in CH_4 by the *methanogenic* bacteria would have been generated through acetate and butyrate production (BAKER, 1999).

The estimated amounts of CH_4 produced per mg OM degraded during 48 h of fermentation were 0.07, 0.09 and 0.13 mole respectively, for Gliricidia, Leucaena and Calliandra (Figure 7). Using these estimated values and the degradability values of the three shrub legumes (68%, 63% and 49% for Leucaena, Gliricidia and Calliandra, respectively), the total amount of CH_4 released by the animals fed these feeds might be estimated. For the animals fed 500 g of OM, the CH_4 released by animals fed Gliricidia, Leucaena and Calliandra would be 22, 30 and 32 mole. Thus among the three shrub legumes examined, Leucanea produced the lowest CH_4 followed by Gliricidia and Calliandra.

The estimated CH_4 produced in the current experiment was in the range of CH_4 production measured in sheep fed high protein diet such as oat hay (28 mole/g 500 g DMI; CHANDRAMONI *et al.*, 2002) and sheep fed high fiber diet such as *Lotus pedunculatus* (45 mole/500 g DMI; WOODWARD, *et al.*, 2001). The lower yields of CH_4 released from animals fed high protein diet (the three shrub legumes examined) would have important implications, not only in relation to the efficiency of use of food energy by the ruminant

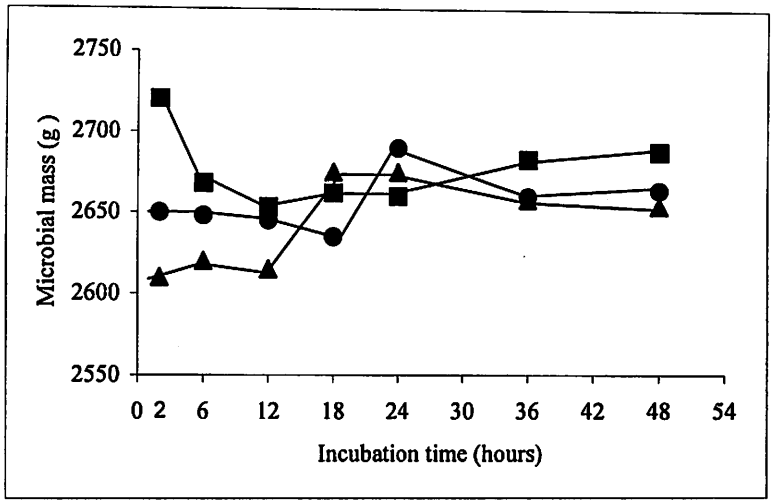

Figure 8. Estimation of microbial cells produced from Leucaena (•), Gliricidia (■) and Calliandra (▲) degraded during the 48 hour of incubation

animals but also to the greater environmental issue in which it is estimated (HARPER *et al.*, 1999) that about 16% of total CH_4 is released to the atmosphere.

Microbial protein synthesis

The estimated values of microbial cells produced from the feeds fermented in the culture bottle (see Figure 8) suggests higher microbial growth on the Gliricidia than on the Leucaena and Calliandra during the first 2 h of the incubation period. By 48 h of incubation the differences in microbial growth among the legumes were minimal.

The amounts of microbial cells from the legumes remained relatively stable and did not appear to increase as the concentration of NH_3 increased. This might be explained by the observation that there was no matching increase in VFA in these feeds to provide appropriate NH_3: VFA ratios for optimum microbial growth. This observation agreed with the work of VOLDEN (1999), that the microbial cells synthesised declined by 19% when the ratio of NH_3: VFA in the rumen of cows increased from 0.04 to 0.09.

CONCLUSION

Among the three shrub legumes examined, Gliricidia has the highest amount of OM degraded (505 mg) and microbial cell and NH_3 produced (2676 g and 1.91 mmol/100 mL) than followed by Leucaena (423 mg; 2656 g; 1.61 mmol/100 mL) and Calliandra (340 mg; 2644 g; 1.61 mmol/100mL). However Gliricidia produced the lowest amount of gas total and CH_4 (0.192 mL and 0.07 mole per mg OM deraded) then Leucaena (0.249 mL and 0.097 mole per mg OM degraded) and Calliandra (0.196 mL and 0.126 mole per mg OM degraded). In the energetic efficiency for the animals fed these shrub legume, therefore, Gliricidia more efficient in term of using the dietary energy for the animals' production compared to the Leucaena and Calliandra.

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