Comparison Fermentation Kinetics (*In Vitro*) of Grass and Shrub Legume Leaves: The Pattern of VFA Concentration, Estimated CH_4 and Microbial Biomass Production

Y. WIDIAWATI and A. THALIB

Balai Penelitian Ternak, PO Box 221 Bogor 16002

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ABSTRAK

WIDIAWATI, Y. dan A. THALIB. 2007. Perbandingan pola fermentasi (*in vitro*) dari rumput dan daun pohon legum: Pola konsentrasi VFA, estimasi produksi CH_4 dan biomasa mikroba. *JITV* 12(2): 96-104.

Degradasi karbohidrat dan protein pakan oleh mikroba rumen menghasilkan produk akhir berupa VFA, protein mikroba, vitamin B, gas CH_4 dan CO_2. Proporsi dari setiap produk akhir ini sangat tergantung pada tipe hijauan yang dikonsumsi ternak. Rumput yang mengandung serat kasar tinggi dan protein yang rendah saat difermentasi oleh mikroba rumen akan menghasilkan produk akhir yang berbeda dengan tanaman leguminosa yang mengandung protein tinggi tapi rendah serat kasarnya. Metoda *in vitro* digunakan untuk mengetahui pola dari produksi VFA, protein mikroba, dan gas CH_4 yang dihasilkan pada saat kedua jenis pakan yaitu rumput dan tanaman leguminosa didegradasi oleh mikroba rumen. Hasil penelitian menunjukkan bahwa rumput menghasilkan lebih banyak VFA total per unit OM yang dicerna (0,0229 mM/mg *vs* 0,0075 mM/mg) dan gas CH_4 (0,20 mol/mg *vs* 0,09 mol/mg) tetapi lebih sedikit propionat dan protein mikroba (2646 g *vs* 2656 g) dibandingkan dengan tanaman leguminosa. Leucaena memproduksi gas CH_4 32% lebih rendah dibandingkan produksi dari rumput saat didegradasi oleh mikroba rumen, yang berarti lebih sedikit energi yang hilang sebagai gas CH_4 dan lebih banyak energi untuk produksi ternak bila bahan ini digunakan.

Kata Kunci: Rumput, Legum, Asam Lemak Terbang, Gas CH_4, Protein Mikroba

ABSTRACT

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In the process of fermentation, rumen microbes normally convert major fractions of carbohydrates and proteins in a feed to useful end-products (i.e. VFA, microbial protein and B-vitamins) and some waste products (i.e. CH_4 and CO_2). The pattern of these end-products depend largely on the fraction contained in the feed eaten by the animal. Two types of feeds, namely grass and shrub legume, leucaena have different fraction proportions. Grass contains more fibre but less protein compared to shrub legumes. Thus in the rumen they might be fermented to produce different pattern of end products. The experiment was conducted in order to examine the pattern of VFA, CH_4 and microbial protein products of the two types of feeds when fermented in the rumen. *In vitro* method was used to determine the pattern of these end products. Results showed that the grass produced more total VFA/mg organic matter degraded (0.0229 mM/mg *vs* 0.0075 mM/mg) and CH_4 gas (0.20 mole/mg *vs* 0.09 mole/mg) but less propionate in partial and less microbial protein (2646 g *vs* 2656 g) compared to the legume. Approximately 32% less CH_4 (per mg OM degraded) would be produced from leucaena compared to that produced from grass, which mean that there will be less energy loss as CH_4 thus more energy for animal production.

Key Words: Grass, Shrub Legume, Volatile Fatty Acids, CH_4, Microbial Protein

INTRODUCTION

The proportion of major partial of VFA concentration, namely acetate, propionate and butyrate in the rumen depends largely on the type of feed consumped by the animal; in particular, the fractions contained in the feed. Fermentation of a feed containing a large cell-wall or cellulose fraction, is likely to produce a higher molar proportion of acetate (e.g., 70%) and a lower proportion of propionate (e.g., 20%). For plant materials in which cell content is high while the cell wall is low, rumen fermentation would be expected to result in a reduction in the molar proportion of acetate (e.g., 60%) and an increase molar proportion of propionate (e.g., 30%) (DOUGHERTY, 1984).

The synthesis of acetate in the rumen results in an increase production of hydrogen. This also results in an increase activities of *methanogenic* bacteria which uses the hydrogen and the CO_2 produced to synthesize CH_4 (WOLIN *et al.*, 1997). It would follow therefore, that feeds resulting in increase production of acetate and CO_2 would promote an increase CH_4 production, which

would represent a net loss of feed energy as well as inefficiency in feed utilization. The CO_2 produced from carbohydrate fermentation may contribute about 40% of total gas produced in the rumen (MCDONALD *et al.*, 1995); therefore, a feed containing a large amount of carbohydrate should produce more gas than a feed containing less carbohydrate.

This second report describes the differences in both VFA partial (namely acetate, propionate and butyrate) and total concentration, CH_4 and protein microbial production of grass and shrub legume leaves.

MATERIALS AND METHODS

Experimental design

Fermentation kinetics (*in vitro*) of two feeds, namely leucaena and elephant grass, as treatments that were replicated three times, were examined using a Completely Randomised Design (DANIEL, 1991). Each sample was incubated at the periods of 2, 6, 12, 18, 24, 36 or 48 hours.

Feed Samples

Fresh feeds were dried in a freeze-drier (Dynavac Freeze Drying Unit, UK) for about 4 days then were ground to pass through a 1 mm screen (Single Phase A. C. Industry Motor type JY2A-4, West Germany). Each ground feed sample was stored in a plastic bag at 4 °C before being used in the experiment.

The CP, neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents of the experimental feeds were presented in Table 1.

Table 1. Crude protein (CP), acid detergent fibre (ADF), neutral detergent fibre (NDF) and organic matters (OM) in leucaena and elephant grass used as substrate in the current experiment

Nutrients	Treatments	
	Leucaena	Elephant grass
CP (%)	22	10
ADF (%)	28	49
NDF (%)	48	75
OM (%)	89	84

Microbial inoculum

Microbial inoculum was derived from rumen fluid taken from 4 head of rumen-fistulated sheep fed a mixed diet of elephant grass and the leguminous leaves (56:44) at 500 g dry matter (DM)/sheep/day. The composition of the mixed diet was designed to provide a crude protein (CP) content of 13% on a DM basis. This level of CP is recommended for maintenance of tropical sheep (DJAJANEGARA *et al.*, 1996). The ration was divided into two equal portions, one of which was offered at 0800 h and the other at 1600 h. The inoculum was collected at three hours after feeding when the rumen microbes were considered to be at maximal activity (CAMERO and FRANCO, 2001).

Incubation medium

The *in vitro* method developed by THEODOROU and BROOKS (1990) was employed in the experiment. The method used materials namely feed samples as substrates, pooled rumen fluid as the microbial inoculum and a formulated solution as the incubation medium. The medium consisted of six reagents, namely micro-mineral (0.1%), buffer (22.2%), macro-mineral (22.2%), resazurine (0.2%), distilled water (51%) and a reducing agent (4.3%).

Sampling and recording

Organic matter degradation

The feed organic matter (OM) degradation was determined by vacuum filtration through a pre-weighed crucible (Sintaglass, porosity, 70 mL capacity; Gallenkamp, Loughborough, UK). The crucibles containing feed residues were dried in an oven at 100°C for 24 hours or to constant weight to determine DM content, then were ashed in a muffle furnance at 500 °C for 6 hours for OM determination. Organic matter was calculated by subtracting ash from dry matter.

VFA concentration

Each sample of 5 mL aliquots of the medium was transferred into a 10 mL plastic storage vial for the determination of VFA concentration. The concentration of acetate, propionate and butyrate in each sample was determined using Gas Liquid Chromatography (GLC; Hewlett Packard, 3700, USA).

Calculation

Organic matter degradation

The amount of OM degraded was calculated by using Equation: OM degraded = OM in feed sample - OM in residue in each incubation bottle.

Volatile fatty acids concentration

Volatile fatty acids concentration in the solution from each bottle was calculated based on the peak of the graph recorded from the GLC.

$$VFA\ (mmoel) = (P_{spl} / P_{std}) \times (VFA_{std})$$

Where: P_{spl} is height (mm) of the graph recorded when the sample was read by GLC, P_{std} is height (mm) of the graph recorded when the VFA standard was read by GLC and VFA std is the concentration (mmoel) of VFA of the standard solution.

Estimation of methane production

Methane produced during fermentation of the feeds in the culture bottles was estimated using the equation, based on VFA proportions, described by OWENS and GOETSCH (1988):

$CH_4 = 0.5 \times (A) + 0.5 \times (B) - 0.25 \times (P)$

Where CH_4 is amount (mmole) of methane produced, (A) is the concentration (mmole) of acetate, (B) is the concentration (mmole) of butyrate, (P) is the concentration (mmole) of propionate.

Estimation of microbial biomass

OWENS and GOETSCH (1988) suggested that microbial protein synthesised in the rumen from carbohydrate fermentation might be predicted from the value of ATP produced and the values of ATP required for synthesis of a unit of microbial cell. The values of ATP could be estimated from the concentrations of the VFA using Equation :

$ATP_{pr} = 2.5 \times (A) + 2.75 \times (P) + 3.5 \times (B)$.

Where: ATP_{pr} is the amount (mole) of ATP produced, (A) is the concentration (mole) of acetate, (P) is the concentration (mole) of propionate, (B) is the concentration (mole) of butyrate.

With the assumption that each mole ATP produced 10 g of microbial dry cell (OWENS and GOETSCH, 1988), therefore the microbial mass can be estimated.

Microbial mass = $10 \times ATP_{pr}$, where Microbial mass is the amount (g) of microbial cell produced.

Statistical analysis

All raw data were tabulated and analysed using software Excel 2000 and SPSS Version 7.0. The data were analysed using one-way ANOVA (DANIEL, 1991) for Completely Randomised Design. When significant effects from treatments were observed, differences among mean values were examined using Tukey's test (STEEL and TORRIE, 1980)

RESULTS AND DISCUSSION

Nutrient content of feeds used in the experiment

Table 1 showed that the legume leaves contained less cell-wall fraction, as indicated by lower ADF (28%) and NDF (48%) values compared to corresponding values from grass (49 and 75%). The elephant grass contained the highest NDF and the lowest CP per unit of DM.

Organic matter degradation

Data on the amount of OM degraded during the incubation period in the inoculated bottles are presented in Table 2.

The data showed that the amount of OM in leucaena which was degraded after 24 and 48 hours of incubation was significantly higher than that of elephant grass. These results were inverse to the cell wall content of the two type of feeds examined as shown in Table 1, that the ADF and NDF contents of the grass (49 and 75%) were higher than those of leucaena (28 and 48%). It is clearly shown that the structure differences in the cell wall of the legume and the grass contribute significantly to the extent of the difference in the amount of OM degraded between the two types of feed.

These results were similar to the work of MINSON (1990) that grasses or other fibrous feeds, contain high cell wall but less cell content, while most of the shrub legumes would have larger cell content. Some studies showed that the legume contains less NDF about 31 – 47% (AHN *et al.*, 1989) compared to elephant grass (60-75%; KARIUKI *et al.*, 2001). Moreover, WILSON and HATFIELD (1997) reported that cell wall of legume usually does not undergo lignifications as that occurs in the cell wall of grass. Thus the legume diet is easier to be degraded by rumen microorganisms compared to the

Table 2. Means of organic matter (OM) degraded during 48 hours of incubation period from leucaena and elephant grass

OM Degraded (mg)	Treatment			
	Leucaena	Elephant Grass	SE	P
24 h	401[a]	380[b]	2.84	0.01
48 h	452[a]	426[b]	4.48	0.01

Within rows, means with different superscripts differ significantly (P<0.01)

grass. As reported by POPPI and NORTON (1995) that cell wall fraction of plants is degraded slowly in the rumen and is more resistant to rumen microbial degradation when lignifications occurs.

Volatile fatty acids production

The patterns of means of VFA concentration is presented in Figure 1. The concentrations of VFA for the legume at 2 hours of incubation period, was 3.3 mM compared to the value of only 1 mM for the grass (Figure 1). From 2 h of incubation the VFA concentration for the legume gradually decreased while that for the grass increased sharply.

The patterns of VFA concentration during the incubation period were different between the legume and the elephant grass (Figure 1). During the incubation period, the significant differences in VFA concentration between the legume and the grass were recorded at 2 h; 6 h and 36 h of incubation period ($P<0.01$), while the differences were not significant at 12; 18; 24; 30; 42 and 48 h. The rapid increase in VFA concentration in the two types of feeds in the first 2 h of incubation is indicative of the rapid rate of fermentation during this period ensuring due to the presence of the water-soluble fractions of these feeds (MINSON, 1990; SALAWU *et al.*, 1997). The differences in VFA concentrations between the legume and the grass also are indicative of the differences in the amounts of water-soluble fractions between the two groups of feed, namely grass (19%) and legume (26%) and its slow rate of OM degradation for the grass (0.0545 mg/h) compared to the legume (0.0607 mg/h).

It is also shown in Figure 1 that the peak concentrations of VFA were achieved at 2 h and 36 h of incubation for leucaena and elephant grass, respectively. In earlier *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 ruminant animals received alfalfa and wheaten hay, respectively.

The pattern of means of VFA concentration per unit of feed OM degraded is presented in Figure 2.
The elephant grass had the higher VFA concentration per unit of OM degraded during the 48 h of incubation period (Figure 2). The largest difference in concentration between the grass and the legume was observed at 2 h ($P<0.01$) and 6 h ($P<0.01$) of incubation. The significant differences of VFA concentration also recorded at 18 h ($P<0.05$) and 36 h ($P<0.05$) of incubation time. In general, the concentration of VFA per unit of OM degraded in the feeds examined reached maximum at incubation times between 2 h and 6 h.

The higher VFA concentration per unit of OM degraded in the grass than that of in the legume (i.e. 0.0229 mM/mg *vs* 0.0075 mM/mg) most likely was due to the difference in cell wall content of the feeds. Fermentation of the higher carbohydrate (largely structural) constituent in elephant grass produced more VFA, while fermentation of protein in the legume released more NH_3 but less VFA (BLUMMEL and BULLERDIECK, 1997).

The sharp increase followed by a rapid decline in VFA concentration per unit of OM degraded in elephant grass (see Figure 2) most likely were due to the rapid

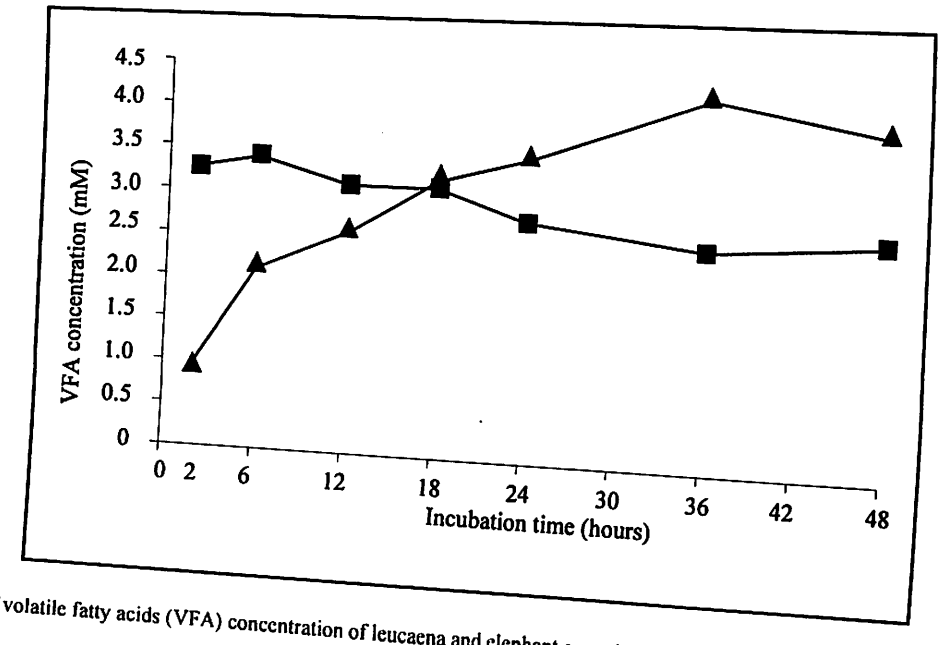


Figure 1. Means of volatile fatty acids (VFA) concentration of leucaena and elephant grass (▲) during the 48 hours of incubation

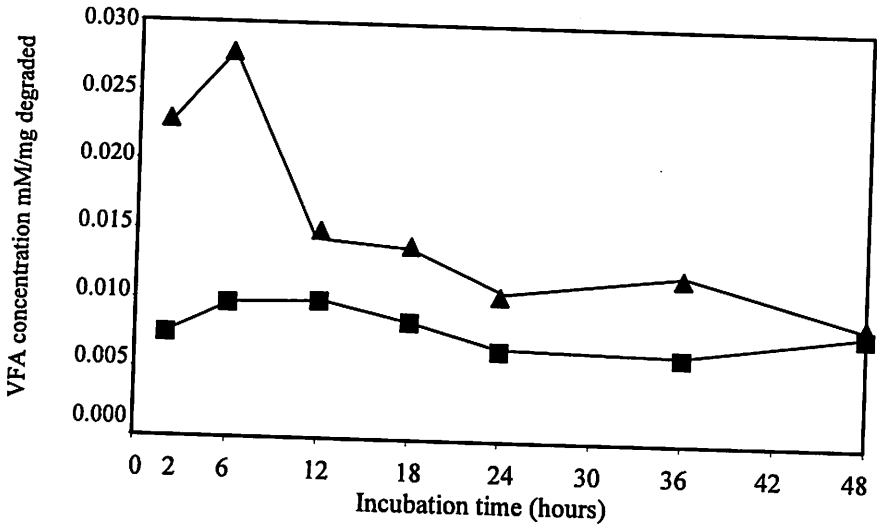


Figure 2. Means of volatile fatty acid (VFA) concentration per unit of organic matter (OM) of leucaena and elephant grass (▲) degraded during the 48 hours of incubation

fermentation of water-soluble fraction containing starch and soluble carbohydrates together with the more easily accessible and degradable cellulose. The grass was high in NDF content (75% of DM; see Table 1) and low in rate of production of VFA (as might be reflected by VFA concentration, Figure 1) probably due to its high NDF content.

SMITH and OLDHAM (1981) observed that the water-soluble fraction of a highly fibrous feed incubated in the rumen was fermented rapidly during the first 5 h of incubation. The cellulose fraction was fermented at a slow rate during this time but started to be fermented more intensively after 5 h of incubation. The data observed in the current experiment would agree, generally, with this finding. It also might be pointed out here that since the observations described in the current experiment were derived from closed *in vitro* systems, in which for one, there was no clearance of the fermentation end-products, VFA. From the culture bottles, the concentration of these acids from the feeds examined might have been under estimated. The relative of VFA concentration among the feeds most probably are correct.

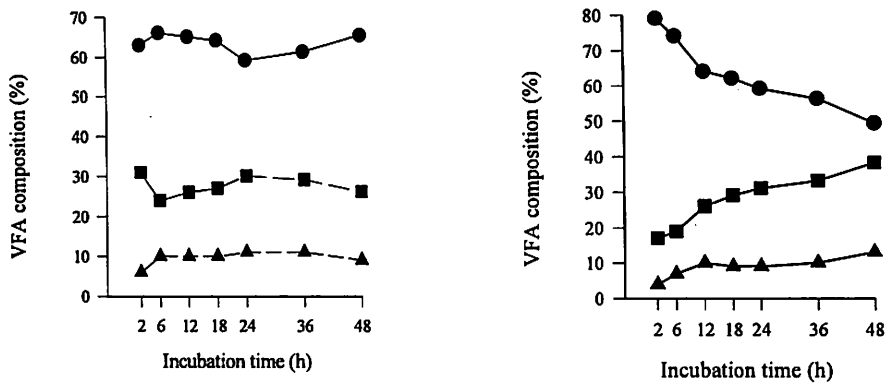
The mean molar proportions of the three main VFA; acetate, propionate and butyrate, in the medium culture bottles during 48 h of incubation are presented in Figure 3. Overall, the molar proportions of VFA in the medium were different between the legume and grass. The figures showed an apparent inverse relationship between the molar proportions of acetate and propionate for the two types of feeds. This relationship is particularly obvious in elephant grass, where the molar proportion of acetate decreased while that of propionate increased sharply. The leucaena had higher proportions of propionate throughout most of the incubation period (Figure 3).

The high cell wall content of the elephant grass as expressed by ADF and NDF content (49 and 75% *vs* 28 and 48%) also would explain the difference between the grass and the legume in the proportions of acetate, propionate and butyrate in the VFA concentration during the period of incubation. Fermentation of cellulose, the major component of the cell wall, would result in proportionally higher acetate concentration and CO_2 release. Therefore, fermentation of a large part of the cell wall in elephant grass would yield more acetate and CO_2. While, the large proportion of propionate concentration in the legume than in elephant grass suggests that more cell content were being fermented (ZARLING and RUCHIM, 1987). These results are in agreement with the observations reported by KEIR *et al.* (1997) and PEREZ-MALDONADO and NORTON (1996). The reduction in the proportion of acetate with a concomitant increase in the proportions of propionate and butyrate observed in elephant grass (Figure 3) was probably as a result of a conversion of acetate to propionate and butyrate. As reported by CHURCH (1976) that in animals fed grass hay, about 14% of propionate was derived from acetate, while only 4% of propionate was formed from acetate in animals fed the legume, alfalfa.

Methane production

Estimated values of CH_4 produced per unit of feed OM degraded in the culture bottles (Figure 4) were almost twice as high as grass than that of the legume during the first 12 h of the incubation period. The significant differences of CH_4 produced between the two feeds examined was recorded at 2 h ($P<0.01$) and 6 h ($P<0.01$) of incubation time.

This would have been expected since the fermentation of grass yields a higher rate of production of acetate (Figure 3b), which was associated with a higher rate of production of CO_2; which in the presence of hydrogen, formed CH_4.



(a) leucaena (b) elephant grass

Figure 3. Mean molar proportion of volatile fatty acid (VFA) occurring as acetate (●), propionate (■), and butyrate (▲) from (a) leucaena and (b) elephant grass during the 48 hours of incubation.

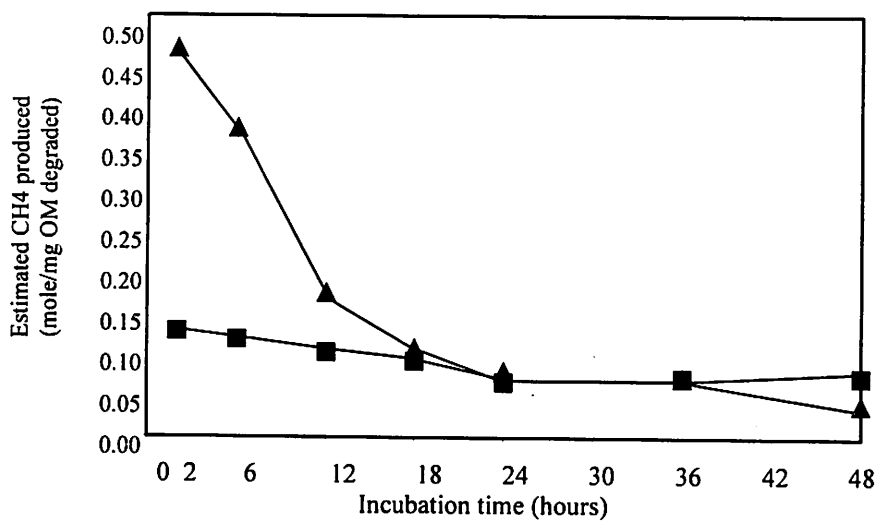


Figure 4. Estimation of methane (CH_4) produced, which was obtained from the concentration of acetate, propionate and butyrate when leucaena and elephant grass (▲) were degraded during the 48 hours of incubation

The net hydrogen produced that would have been disposed of CO_2 in CH_4 by the *methanogenic* bacteria would have been generated through acetate and butyrate production (BAKER, 1999). It should be noted that such clear difference in CH_4 production between the grass and the legume feeds resulted from difference in the rate of fermentation process in the rumen expressed as OM digestibility basis.

The estimated amounts of CH_4 produced per mg of OM degraded during 48 h of fermentation were 0.09 and 0.20 mole respectively, for leucaena and elephant grass (Figure 4). Using these values and the degradability values of the feeds, the total amount of CH_4 released by the animals fed these feeds can be estimated. For example, given an OM intake of 500 g, the CH_4 released by animals fed leucaena and elephant grass would be 30 and 45 mole, respectively.

The estimated CH_4 produced in the current experiment was in the range of data for sheep fed oat hay (28 mole/500 g DMI; CHANDRAMONI *et al.*, 2002) and sheep fed *Lotus pedunculatus* (45 mole/500 g DMI; WOODWARD, *et al.*, 2001). The lower yields of CH_4 released from animals fed the legume would have important implications, not only in relation to the efficiency of use of food energy by the ruminant animals but also to the greater environmental issue in which it is estimated that about 16% of total CH_4 is released to the atmosphere (HARPER *et al.*, 1999).

Microbial protein

The estimated values of microbial cells produced from the feeds fermented in the culture bottle (Figure 5) suggests higher microbial growth on the legume than on the grass feed during the first 2 h of the incubation period ($P<0.05$). This is consistent to the pattern of OM degradation (Table 2). However, during the last 12 h of the incubation period (Figure 5) microbial growth on the grass feed appeared to have outstripped microbial growth on the legume feed.

It is likely that the difference between the grass and the legume in the microbial cells produced was due to the difference in the balance between ammonia nitrogen (NH_3-N) and energy derived from VFA. In the grass, the value of NH_3:VFA (mmole:mmole) observed at 2 h of incubation time was 0.54. This value was associated with the smallest amount of microbial cells synthesised. When the value declined from 0.54 to 0.31 at 48 h of incubation, the amount of microbial cells synthesised was largest for the elephant grass ($P<0.05$). On the other hand, the corresponding value for the legume was 0.52 during 48 h of incubation.

The amounts of microbial cells from the legume 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 values for optimum microbial growth. This

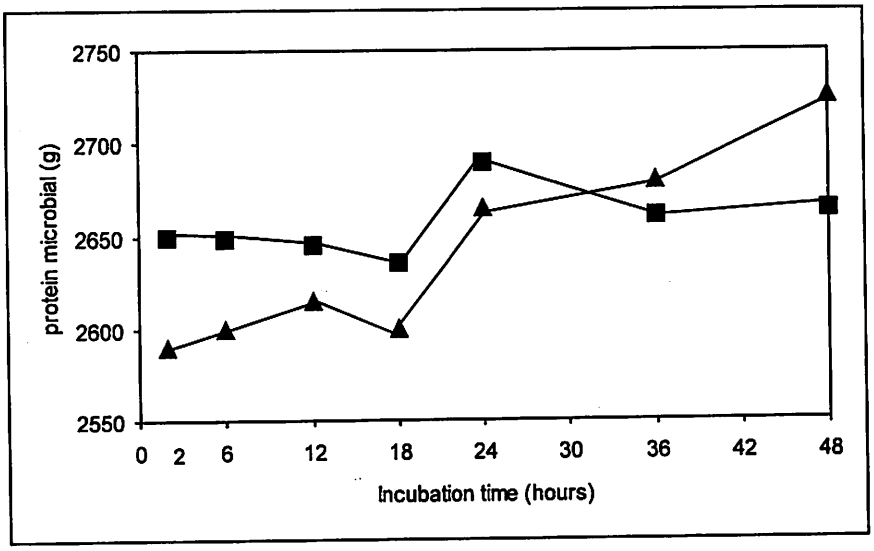


Figure 5. Estimated of protein microbial produced when leucaena and elephant grass (▲) degraded during the 48 hour of incubation

observation agreed with the work of VOLDEN (1999), that the microbial cells synthesised declined by 19% when the value of NH_3:VFA in the rumen of cows increased from 0.04 to 0.09.

CONCLUSION

The amounts of OM degraded and microbial protein produced during fermentation were larger in the leucaena than in the grass. The grass produced more total VFA per unit OM degraded (0.0229 mM/mg *vs* 0.0075 mM/mg) and CH_4 gas (0.20 mole/mg *vs* 0.09 mole/mg) but less propionate in partial and less microbial protein (2646 g *vs* 2656 g) compared to the legume. For the potential environmental implication approximately 32% less methane (per unit OM degraded) would be produced from leucaena compared to that produced from grass, which means that there will be improvement in efficiency in ruminant animal productivity

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