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# *In vitro* Gas and Methane Production of Horse Grass and Calopo at Different Forage to Concentrate Ratios in Rabbits

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author UKO designed the study, conducted the experiment and performed the statistical analysis. Author SNU supervised the experiment. Author MOO drafted the manuscript and managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

The *in vitro* gas production technique provides information about kinetics and fermentation characteristics of feedstuffs and diets, and it is a useful indicator of fecal microbial activity.
**Aim:** The aim of this study was to use the *in vitro* technique to study gas production kinetics and dry matter degradability of feed samples at different forage to concentrate (F:C) ratios using rabbit inoculum.
**Materials and Methods:** The treatments (T) were in a Completely Randomized Design with two forage sources namely Horse grass and Calopo mixed at 75:25 ($T_1$) ratio and F:C ratios 75:25 ($T_2$), 50:50 ($T_3$), 25:75 ($T_4$) and 100:0 ($T_5$), respectively. Six New Zealand White rabbits (mean body weight [BW] = 0.86kg ±0.04) were used as inoculum donors.
**Results:** Crude protein of forages was 10.45 and 23.00 for Horse grass and Calopo respectively.

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The crude protein and crude fiber content of the supplementary concentrate was 17.01 and 6.00% respectively. Highest crude fiber (12.00) was also observed in Calopo. Diets 4 and 5 produced the highest (P<0.05) total gas (ml/200mgDM) and methane (ml/200mgDM) respectively, while *in vitro* degradability decreased with increasing supplementary concentrate (P<0.05). Sole forage diet resulted in highest dry matter degradability (6.90) and gas production (7.80).
**Conclusion:** It can be concluded that good quality forage, therefore, could reduce the need for concentrate supplementation.

*Keywords: Caecal fermentation; degradability; gas kinetics; nutrient; tropical forages.*

## 1. INTRODUCTION

The rabbit (*Oryctolagus cuniculus*) caecum represents 40% of the total digestive tract by weight and accounts for about two-thirds of fecal fiber digestion [1]. It is characterized by the presence of an abundant microbial community with wide diversity and complex interactions [2]. Microbial community plays a role in feed digestion through fermentation and recycling of microbial protein through caecotrophy. Grasses [3] and legumes such as *Calopogonium mucunoides* [4] have been reported to be acceptable to rabbits. Though rabbits have been found to perform best on concentrate [5], the high cost of grains has caused animal scientists to shift to forages which are cheap and available. The *in vitro* gas production technique has been used to evaluate the nutritive value of feed in caecal fermenters. It also provides information about kinetics and fermentation characteristics of feedstuffs and diets, and it is a useful indicator of fecal microbial activity [6]. Therefore, the aim of this experiment was to investigate the effects of grass: legume mixed forages combined with different forage to concentrate ratios on dry matter degradation and gas production kinetics using the *in vitro* technique.

## 2. MATERIALS AND METHODS

### 2.1 Substrate Preparation and Analysis

Prior studies were carried out with rabbits to determine the acceptability of various forages. Horse grass and Calopo (75:25) were the most desirable and also improved performance. This informed the decision to use forages and concentrates as substrates as follows: sole forage ($T_1$: 75% Horse grass + 25% Calopo), Three concentrate to forage ($T_2$: 25% concentrate + 52.5% Horse grass + 22.5 Calopo; $T_3$: 50% concentrate + 35% Horse grass + 15% Calopo; and $T_4$: 75% concentrate + 17.5% Horse grass + 7.5 Calopo) and sole concentrate diet ($T_5$: 100% concentrate). Substrates were sub-sampled and initially weighed fresh on the field and then oven-dried to 65ºC to constant weight. Dried samples were weighed and hammer-milled to pass through a 1mm sieve. Crude protein (% Nitrogen x 6.25), crude fiber, ash and ether extract were analyzed according to the standard methods of Association of Official Analytical Chemists [7].

### 2.2 Animal Donors and Collection of Caecal Fluid

Six New Zealand White rabbits (mean body weight [BW] = 0.86kg ±0.04) were used as inoculum donors. The animals were previously fed commercial growers mash. Caecal fluid was collected in the morning in equal proportions from donor animals, under the same feeding regime post-slaughtering. Caecal contents collected from gastro-intestinal tract isolated after slaughtering were emptied into a pre-warmed thermos flask and strained through four-layered cheesecloth and kept at 39ºC soon after collection. All laboratory handling of caecal fluid was carried out under a continuous flow of carbon dioxide ($CO_2$). The inoculum and a buffer solution were mixed in the ratio 1:2 (v/v), respectively as described by Menke and Steingass [8].

### 2.3 *In vitro* Procedure

Oven-dried samples (0.2kg) were milled and accurately weighed into 100ml gas syringes fitted with plungers. *In vitro* incubation of the samples was conducted in triplicates. Syringes were filled with 30ml of medium consisting of 10ml of caecal fluid and 20ml of buffer solution. Three blanks containing 30ml of medium were incubated. The syringes were placed in rotor inside the incubator (39ºC) with about one rotation per minute. The gas production was recorded after 3, 6, 9, 12, 18, and 48 h of incubation. The volume of methane gas produced by each diet was determined by

dispensing 4ml of 10 N sodium hydroxide (NaOH) into each incubated sample at the end of the 48 h of incubation. NaOH was added to absorb carbon-dioxide produced during fermentation and the remaining volume of gas recorded as methane as described by Fievez et al. [9].

### 2.4 Dry Matter Degradation, Organic Matter Digestibility, and Metabolizable Energy

The *in vitro* dry matter degradation was determined at 48 h by centrifuging the incubation residues at 20, 000 x g for 30 minutes following placement in the iced cube (-4ºC) to end fermentation. Residues obtained were filtered and oven-dried to determine their dry weight. The blanks were also centrifuged, and residues weighed and used to correct for residues from caecal 275 inoculums. *In vitro* dry matter degradation was then calculated as:

(Weight of sample before incubation – Weight of sample after incubation / Weight of sample after incubation. Organic matter digestibility (OMD) and metabolizable energy (ME) was determined using the following equations as described by Menke and Steingass [8]. OMD = 14.88 + 0.88GV + 0.45CP + 0.651XA. ME was determined using the equation: ME = 2.20 + 0.136GV + 0.057CP + 0.00029CF (Menke and Steingass, 1988). Where GV is total gas volume produced, CP is crude protein and CF crude fiber.

### 2.5 Data Analysis

All data obtained were subjected to Statistical Analysis System [10] using a completely randomized design. For all parameters, differences between treatment means were contrasted by Duncan's new multiple range test [11].

## 3. RESULTS

### 3.1 Chemical Composition of Experimental Diets

At 45 days of growth, the dry matter, crude protein and crude fiber content (Table 1) of Horse grass and Calopo were 27.33, 10.45, 10.00% and 30.67, 12.00 and 10.00% respectively. The concentrate diet had 17.01 and 6.00% CP and CF respectively. Horse grass and Calopo had the same (7.00%) ash content.

### 3.2 Gas Production Kinetics and *in vitro* Digestibility

Gas production kinetics is shown in Table 2. They were significantly affected by diets. Gas production level at 48 h was highest ($P<0.05$) in the diets with forage:concentrate (F:C) proportion of 75:25 ($T_4$) and lowest in 25:75 F:C ratios ($T_2$).

**Table 1. Feed ingredients and chemical compositions of concentrate and forages**

| Feed ingredients | Percentage | Horse grass | Calopo |
|---|---|---|---|
| Maize offal | 54.00 | | |
| Palm kernel cake | 20.00 | | |
| Wheat offal | 10.00 | | |
| Soybean meal | 10.00 | | |
| Fish meal | 2.50 | | |
| Bone meal | 3.00 | | |
| Vitamin-mineral premix | 0.25 | | |
| Common salt | 0.25 | | |
| **Total** | **100.00** | | |
| Dry matter (%) | 92.24 | 27.33 | 30.67 |
| Crude protein (%) | 17.01 | 10.45 | 12.00 |
| Crude fiber (%) | 6.00 | 10.00 | 10.00 |
| Ether extract | 5.30 | 4.10 | 5.70 |
| Ash (%) | 7.00 | 7.00 | 7.00 |
| Nitrogen-free extract (%) | 64.69 | 68.45 | 75.20 |

**Table 2. Gas production kinetics and *in vitro* degradability**

| F:C ratios | Gas | Production | Kinetics | | Total gas production (mL/ 200 mg DM) | *In vitro* degradability | OMD (%) | ME (MJ/ kg DM) |
|---|---|---|---|---|---|---|---|---|
| | A | B | a+b | C | (48 h) | IVDMD (%) | | |
| 100:0 | 1.83$^{a}$ | 5.97$^{c}$ | 7.80$^{b}$ | 0.04 | 7.80$^{b}$ | 6.90$^{a}$ | 31.87$^{a}$ | 4.48$^{a}$ |
| 75:25 | 1.00$^{b}$ | 5.10$^{c}$ | 6.10$^{c}$ | 0.02 | 6.10$^{c}$ | 5.90$^{ab}$ | 27.30$^{bc}$ | 3.77$^{bc}$ |
| 50:50 | 1.00$^{b}$ | 7.40$^{b}$ | 8.40$^{b}$ | 0.02 | 8.40$^{b}$ | 5.45$^{ab}$ | 26.06$^{c}$ | 3.66$^{c}$ |
| 25:75 | 1.00$^{b}$ | 10.80$^{a}$ | 10.80$^{a}$ | 0.02 | 11.80$^{a}$ | 3.20b$^{c}$ | 28.39$^{bc}$ | 4.01$^{bc}$ |
| 0:100 | 1.00$^{b}$ | 7.20$^{b}$ | 8.20$^{b}$ | 0.02 | 8.20$^{b}$ | 2.20$^{c}$ | 28.77$^{bc}$ | 4.07$^{b}$ |
| SEM | 0.11$^{*}$ | 0.20$^{*}$ | 0.17$^{*}$ | 0.009$^{ns}$ | 0.17$^{*}$ | 0.56$^{*}$ | 0.41$^{*}$ | 0.06$^{*}$ |

*abc: means along the same column with different superscripts are significantly different (P<0.05).*

** = significant; ns = not significant; SEM: standard error of means; a = intercept (gas produced from soluble fraction); b = gas production from insoluble fraction; c = gas production rate constant for insoluble fraction (b); IVDMD = in vitro dry matter degradability; IVOMD = in vitro organic matter digestibility.*

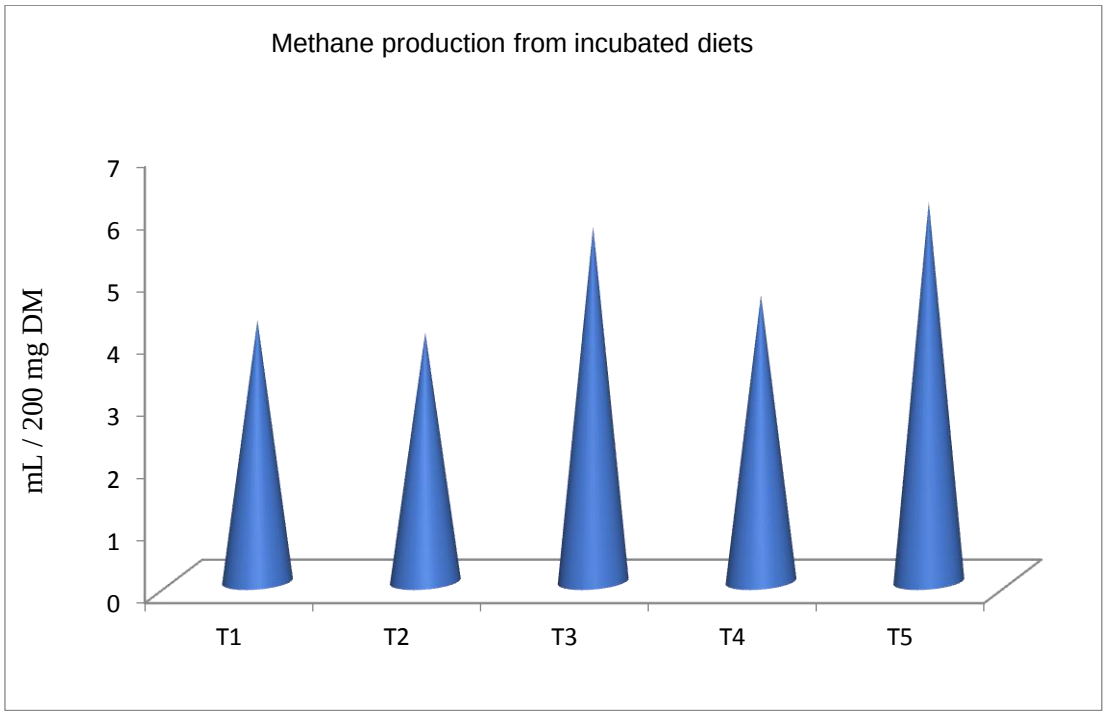


**Fig. 1. Methane production after 48 h incubation**

Methane production from incubated samples (Fig. 1) showed that diets 5 (6.10 mL/ 200 mg DM) and 3 (5.70 mL/ 200 mg DM) produced significantly the highest ($P<0.05$) volume of methane. The soluble fraction 'a' of the samples was significantly higher ($P<0.05$) in diet 1(1.83) while other diets were the same (1.00). Potential gas production 'b' (mL / 200 mg DM) estimated for $T_1$ (5.97) and $T_2$ (5.10) were statistically similar and lower than diets 3 (7.40) and 5 (7.20). The fermentation rate constant 'c' of the insoluble fraction for all diets was not significantly ($P>0.05$) different by treatments, although diet 1 was slightly higher than other diets. Sole forage ($T_1$) diets were highest ($P<0.05$) in IVDMD (6.90) and OMD (31.87). The highest (4.48 MJ/ kg DM) and the least (3.66 MJ/ kg DM) predicted ME was observed in $T_1$ and $T_3$ respectively.

## 4. DISCUSSION

Total gas production values obtained at 48 h in this study are lower than that reported by Khanum et al. [12] but similar to the values reported by Njidda et al. [13]. These values depend to a large extent on the level and nature of fiber, the presence of secondary metabolites and most importantly, the potency of the caecal/ruminal fluid used as inoculum [14]. Generally, in rabbits, caecal microorganisms ferment available nutrients mainly polysaccharides to short-chain fatty acids, ammonia, and gases. The amount of gas produced *in vitro* fermentation is directly related to the amount of short chain fatty acids production [15]. Therefore, by gas production, the diet classification is $D_2 < D_1 < D_5 < D_3 < D_4$ which conforms to the trend of the feeding except for $D_1$ that took the place of D2 in the *in vitro* trial. It has been reported in most cases [16,17] that feedstuffs which show a high capacity for gas production are also observed to be synonymous with high methane production. The results of this study confirmed this assertion. With regards to diet four that produced the highest methane, while fermentable carbohydrates increase methane production, it has been reported that addition of degradable nitrogen sources to forages and fibrous feeds, decreased methane production due to better or improved capturing of nutrients and higher production of microbial protein [8]. This was the case with diet 4 of this trial confirmed by both *in vivo* and *in vitro.* Dry matter degradability values obtained in his study is similar to values reported by Njidda et al. [13] but at variance with values of [12]. This could be as a result of the level and nature of fiber, the presence of secondary metabolites and most importantly, the potency of the caecal/ruminal fluid used as inoculum as reported by Babayemi et al*.* The predicted difference between ME and OMD of diets reflected contents of fermentable

carbohydrate and available nitrogen [8]. Owing to the nutritional adequacy of mixed forage diet, it would be expected that gas production pattern should be higher with increasing amount of forages in the diets, but the reverse was the case in this study. Thus, this confirms the observation of [18] that the presence of nitrogen in forages does not always guarantee its availability to that target microbes in the caecum or rumen.

## 5. CONCLUSION

Sole forage diet resulted in highest dry matter degradability and gas production. It may be suggested that good quality forage could reduce the need for concentrate supplementation. Further research using Horse grass and Calopo to improve caecal fermentation and feed efficiency in rabbits are recommended.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

As per international standard or university standard, written ethical approval has been collected and preserved by the authors.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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