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RESEARCH ARTICLE

Effect of replacement of ordinary ruminant feed with *Hedychium gardnerianum* or *Pittosporum undulatum* on *in vitro* rumen fermentation characteristics

Mohamed A. Moselhy [1, 2,] *, Helder P. Nunes [1] and Alfredo E.S. Borba [1]

1- Animal Nutrition, Department of Agricultural Sciences, University of the Azores, CITA-A, 9700-042 Angra do Heroísmo, Azores, Portugal

2- Microbiology Department, Faculty of Agriculture, Cairo University, 12613 Giza, Egypt

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****Corresponding Author***

Dr. Mohamed A. Moselhy

Abstract

The present study was undertaken to assess the effect of replacement of ruminant feed with stepwise levels (0, 25, 50, 75 and 100%) of *Hedychium gardnerianum* (HG) or *Pittosporum undulatum* (PU) on *in vitro* gas production characteristics, CH_4 production, microbial activities and energy parameters. The assessment was carried out using *in vitro* gas production technique. Cumulative gas production was recorded at 3, 6, 9, 12, 24, 48, 72 and 96 h of incubation. Bacterial and protozoal counts were microscopically estimated. *In vitro* true dry matter digestibility and CH_4 production were also determined by the end of fermentation course. The results showed that the treatment HG25% significantly surpassed (P<0.05) all other treatments in most of investigated parameters, followed by the treatment PU25%. While, treatment HG100% was the least significant one (P<0.05). Cumulative gas production was in a range 24.09 - 49.91 ml 200 mg^{-1}DM whereas, CH_4 production laid in a range 4.48 - 18.71 ml 200 mg^{-1} DM. Total bacterial and total protozoal counts were in ranges 1.92 - 6.23 x 10^8 and 2.0 - 5.17 x 10^5 cell ml^{-1}, respectively. Furthermore, the recorded ranges for short chain fatty acids and metabolizable energy were 0.273 - 0.567 mmol 200 mg^{-1} DM and 4.36 - 6.32 MJ kg^{-1} DM, respectively. The current study suggested that the partial inclusion of the tested plants to the ruminant diet can be a promising strategy for improving ruminant feed efficiency and reducing ruminant feed cost as well.

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INTRODUCTION

Ruminant feeding is mainly based on grazing, specifically of Graminaceae. Grasses yield is in general not enough to satisfy the nutritional requirements of animals in the six months of dry period each year. The dry season causes nutritional stress and consequently decreases animal productivity. As well, supplementation with concentrates during the dry season is not a profitable practice due to high feeding costs (Benavides, 1994). So, a potential strategy for increasing the quality and availability of feeds for smallholder ruminant animals in the dry season may be through the use of fodder trees and shrub forages as non-conventional feed resources (Pezo, 1991).

Ruminants depend on microorganisms to digest and ferment plant cell wall polysaccharides into energy sources, such as volatile fatty acids (VFA) and other organic acids. However, microbial fermentation in the rumen also produces waste products, such as carbon dioxide (CO_2) and methane (CH_4). Methane production in the rumen is an energetically wasteful process that represents a loss of 2–15% of the ruminant's gross energy intake (Moss and Givens, 1993). Also, it is known that CH_4 contributes to climatic change and global warming by trapping outgoing terrestrial infrared radiation

and its role in the destruction of the stratospheric ozone layer is uncontested (Wuebbles and Hayhoe, 2002). Thus, mitigating methane losses from ruminants has bilateral; economical and ecological benefits.

Hedychium gardnerianum Sheppard ex Ker-Gawler is a rhizomatous perennial herb of the Zingiberaceae family, it is known as the Kahili Ginger. It has a stalk which can extend up to 2 m long, with oblong leaves reaching 30 cm and several yellow-orange flowers in a spike of 20–30 cm in length. It is an aggressive invasive weed capable of spreading rapidly and dominating large areas in the Azores (Portugal). Moreover, *H. gardnerianum* out-competes many native plants and has become a significant threat to the survival of many of them (Sjögren, 1984; Medeiros et al., 2003). No use is known for this plant, apart the ornamental one and, possibly, the protection of steep hillsides from erosion (Carvalho et al., 2003). *Pittosporum undulatum*, Vent., is a tree or shrub of the Pittosporaceae family. It has white flowers and lanceolate, acute, glabrous leaves with undulated margins. *P. undulatum* was introduced into the Azores from Australia long ago (Sjögren, 1984; Medeiros et al., 2003). The presence of this species in the Azores has created a large problem in biodiversity and decrease the native species richness due to its high reproduction capacity (Silva and Smith, 2006). In the Azores archipelago, *P. undulatum* is used as a source of wood for firewood and for carving. Recently, many of biological activities have been approved for the secondary metabolites extracted from both plants, such as; insect control, molluscicidal effect, antioxidant properties and antimicrobial activities, etc. (Medeiros et al., 2003; Rosa et al., 2010; Teixeira et al., 2012).

In fact, there is a lack of information regarding the *in vitro* gas production, kinetics of gas productivity and the nutritive value of *H. gardnerianum* and *P. undulatum*. In addition, there was no idea about if these plants have any potential to reduce the extent of CH_4 production or not. Thus, the objectives of the current work were to investigate the nutritive value and to study the ruminal fermentation characteristics of *H. gardnerianum* and *P. undulatum* as alternatives for ruminant feed using the *in vitro* gas production technique. As well, to determine if the addition of these plants to the ruminant diet could influence the amount of CH_4 that is produced during the ruminal fermentation.

MATERIALS AND METHODS

Collection of plant materials: Two plants namely *Hedychium gardnerianum* and *Pittosporum undulatum* were under investigation. The whole plant materials (leaves, stems, flowers and fruits) except roots were collected from forests around Angra do Heroismo city, Terciera, Azores, Portugal. Samples were chopped into small pieces, then dried at 55ºC in a forced air oven for 72 h, ground to pass a 1mm sieve using a Retsch mill (GmbH, 5657 HAAN, Germany) and stored in tightly closed bags till use.

Chemical analysis: Chemical analysis was in triplicate. Dried samples were subjected to analyze dry matter (DM), crude protein (CP), ether extract (EE) and total ash according to the standard methods of AOAC (1995). Briefly, the dry matter content in feed was determined by placing a feed sample in a forced air oven at 105°C for 24 h and then DM content was calculated. Total ash was evaluated by igniting the samples in a muffle furnace at 600°C for 12h. Crude protein was determined by standard micro-Kjeldahl method using digestion equipment (Kjeldatherm System KT 40, Gerhart Laboratory Instruments, Bonn, Germany) and an automated Kjelfoss apparatus for distillation and titration (Foss Electric, Copenhagen, Denmark). Ether extract was assessed by refluxing feed samples with petroleum ether in a soxhlet system (Büchi B-810, Switzerland). Where, neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined according to Van Soest et al. (1991). Both NDF and ADF were expressed without residual ash. Moreover, organic matter content was calculated as 100 – %ash. Hemicellulose and cellulose were calculated as NDF – ADF and ADF – ADL, respectively. Finally, Non-fiber carbohydrates (NFC) were estimated as NFC % = 100 - (%NDF + %CP + %EE + %Ash) according to NRC (2001). The chemical composition of *H. gardnerianum*, *P. undulatum* and the other used feed materials is displayed in Table 1.

Experimental diets for *in vitro* fermentation: Total nine mixed rations were formulated from both plants (*H. gardnerianum* or *P. undulatum*) and the control feeding diet (40% straw mixture, 40% green grass mixture and 20% commercial concentrate) to include five addition levels of each dried plant (0%, 25%, 50%, 75%, and 100%). Different investigated diets for *in vitro* fermentation with selected chemical composition values are demonstrated in Table 2.

Rumen fermentation and *in vitro* gas production:

Donor animals and rumen liquor collection: Rumen fluid was collected by a stomach tube before the morning meal from 3 rams of Romney March sheep (mean body weight 40 ± 2.0 kg). The sheep were fed on a diet consisted of 40% green grass mixture, 40% straw mixture and 20% commercial concentrate for 21 days before each sampling time. The animals were fed ad libitum and had free access to a clean water source. Ruminal fluid was collected into a pre-warmed insulated flask and immediately transported to the laboratory.

Table 1. Chemical composition of *Hedychium gardnerianum*, *Pittosporum undulatum* and ruminant ordinary diet ingredients.

Parameters*	Composition %				
	Hedychium gardnerianum	*Pittosporum undulatum*	Concentrate	Straw mixture	Grass
Dry matter (DM)	16.34	40.39	87.0	86.25	15.64
Total ash	10.04	6.64	7.40	11.92	12.0
Organic matter (OM)	89.96	93.36	92.6	88.08	88.0
Crude protein (CP)	7.75	6.11	16.17	3.45	18.66
Neutral detergent fiber (NDF)	64.50	43.84	33.20	72.41	49.41
Acid detergent fiber (ADF)	34.69	35.57	14.11	49.53	27.28
Hemicellulose	29.81	8.27	19.09	22.88	22.13
Acid detergent lignin (ADL)	7.03	15.24	6.74	14.41	2.68
Cellulose	27.66	20.33	7.37	35.12	24.60
Ether extract (EE)	2.30	2.71	3.20	0.57	2.69
Non-fibrous carbohydrate (NFC)	15.41	40.70	40.03	11.65	17.24

*All values are expressed on dry matter basis (%), except DM is expressed on fresh matter basis (%).

Table 2. The experimental diet formulations with some selected chemical composition values.

Diet formulation	Diet composition %				
	DM	Ash	CP	C/N ratio*	EE
Control 0% (ordinary feed; 40% straw mixture, 40% grass and 20% concentrate) (C)	94.65	11.90	11.43	27.94	1.90
25% *Hedychium* + 75% ordinary feed (HG25%)	93.77	11.27	10.63	30.26	2.09
50% *Hedychium* + 50% ordinary feed (HG50%)	92.98	11.03	9.30	34.68	2.20
75% *Hedychium* + 25% ordinary feed (HG75%)	92.11	10.60	8.04	40.31	2.26
100% *Hedychium* + 0% ordinary feed (HG100%)	97.43	10.04	7.75	42.09	2.30
25% *Pittosporum* + 75% ordinary feed (PU25%)	93.87	10.50	10.37	31.29	2.38
50% *Pittosporum* + 50% ordinary feed (PU50%)	93.30	9.26	8.80	37.38	2.53
75% *Pittosporum* + 25% ordinary feed (PU75%)	92.71	8.09	7.24	46.02	2.62
100% *Pittosporum* + 0% ordinary feed (PU100%)	97.45	6.64	6.11	55.39	2.71

DM = Dry matter; CP = Crude protein; EE = Ether extract.

*; C/N ratio = Carbon : Nitrogen, where; C = organic matter (OM%) x 0.58, OM = 100 - Ash% (Tiquia and Tam, 1998) and N = CP / 6.25.

Inoculum preparation and *in vitro* incubations: *In vitro* gas production was determined as per the procedure described by Menke and Steingass (1988) using *in vitro* Hohenheim gas test apparatus. In brief, 200 ± 10 mg of samples (Table 2) were accurately weighed into 100 ml calibrated glass syringes with pistons lubricated with Vaseline. Buffered mineral solution (Menke and Steingass, 1988) was prepared and placed in a water bath at 39°C under continuous flushing with CO_2. Rumen fluid was collected as the aforementioned, homogenized and strained through 4 layers of muslin then kept at 39°C under a continuous CO_2 stream. The reducing fluid was prepared and mixed with the buffer. The well-mixed and CO_2-flushed rumen fluid was added to the buffered mineral solution (at a ratio of 1:2 v v^{-1}). The mixture was kept stirred under CO_2 in a water bath at 39°C, using a magnetic stirrer. Buffered rumen fluid (30 ml) was dispensed into each syringe containing the weighed diet samples and immediately placed in a rotor inside the incubator (39°C ± 0.5°C) with about one rotation per min. Three syringes containing only 30 ml inoculum (buffered rumen) were served as blanks.

The incubations were carried out in triplicate for each feed sample and these incubations were repeated three times (3 different runs). In each run, the performed syringes of each treatment were repeated twice in a parallel incubation. One set of syringes (1st set, incubated for 24 h) was conducted for measurement of pH, counting of bacteria and protozoa. Whereas, another replica set (2nd set, incubated for 96 h) was applied in case of gas volume measurements, gas kinetics study, CH_4 and *in vitro* true dry matter digestibility (IVTDMD) determinations.

Post fermentation analyses and calculations:

Total gas production: The total gas production was recorded after 3, 6, 9, 12, 24, 48, 72, and 96 h of incubation (2nd set). The fermentation gas volume was read from the calibrated scale on the glass syringes. The actual total gas production was calculated by subtracting gas produced in blank syringes from total gas produced in syringes containing feed substrates and buffered inoculum.

Methane determination: Methane content in fermentation gas mixture was determined after 96 h of incubation (2nd set) as explained by Demeyer et al. (1988) and Fievez et al. (2005). Immediately after removal of the syringes from the incubator, 4 ml of 10M NaOH were introduced using a 5 ml capacity syringe. The content was inserted into the silicon tube, which was fastened to the 100 ml capacity syringe. The clip was then opened while the NaOH was gradually released. The content was agitated while the plunger began to shift position to occupy the vacuum created by the absorption of CO_2. The volume of methane was read on the calibrated body of the syringe.

Measuring of the pH: The pH values of fermentation fluid samples (1st set) were measured by using a pH digital meter (model 632, Metrohm, Herisau, Switzerland).

Counting of bacteria and protozoa: Total bacterial and protozoal counts in the incubation fluid samples (1st set) were determined after 24 h fermentation period by direct microscopic method using 0.02 mm and 0.1 mm depth Bürker counting chambers (Blau Brand, Wertheim, Germany), respectively. Prior to bacterial counting, samples were fixed (1:10 dilution) with Hayem solution ($HgCl_2$, 2.5 mg ml^{-1}; Na_2SO_4, 25 mg ml^{-1}; NaCl, 5.0 mg ml^{-1}) as reported by López et al. (2009). For protozoal counting, samples were preserved by adding an equal volume (1 ml) of 18.5% (v v^{-1} in water) formaldehyde. Then, three drops of Brilliant green dye were added to 1 ml of this mixture, mixed and allowed to stand overnight. The stained protozoa were diluted if needed with 30% glycerol solution and counted as per the method described by Dehority (1984).

Estimation of *in vitro* true dry matter digestibility (IVTDMD): *In vitro* true digestibility of dry matter was determined according to the procedures of Blummel and Becker (1997). The remaining fermented contents of the syringes (2nd set) were drained into a 600 ml spoutless beaker and syringes were thoroughly washed (3 times) with a total of 100 ml neutral detergent solution (NDS). The contents were refluxed for 1 h in NDS. The feed residue was recovered on a G_2 pre-tared filter crucible, dried at 105°C for 24 h and weighed as described by Van Soest et al. (1991). *In vitro* true dry matter digestibility was calculated as follows:

$$\text{IVTDMD\%} = \frac{\text{DM of feed taken for incaubation (mg)} - \text{NDF residue (mg)}}{\text{DM of feed taken for incaubation (mg)}} X100$$

Gas production kinetics and calculations: Cumulative gas production data were fitted to the model of Ørskov and McDonald (1979):

$p = a + b\,(1-e^{-ct})$

where:

p is the gas production at time t

a = is the gas production from the immediately soluble fraction (ml 200 mg^{-1} DM)
b = is the gas production from the insoluble fraction (ml 200 mg^{-1} DM)
c = is the gas production rate constant for the insoluble fraction (ml h^{-1})
a + b = is the potential gas production (ml 200 mg^{-1} DM)
e = is the exponential function
t = incubation time (h).

The fermentation constants a, b and c were calculated by a fitting curve method using Neway Software program (Rowett Research Institute, Aberdeen, UK) that developed by Chen (1997).

Organic matter digestibility (OMD), metabolizable energy (ME) and net energy (NE) were calculated respectively, from the following equations according to Menke and Steingass (1988):

$OMD (\%) = 14.88 + 0.889*GP_{24} + 0.45*CP + 0.0651*CA$
$ME (MJ\ kg^{-1}\ DM) = 2.20 + 0.136*GP_{24} + 0.057*CP + 0.0029 *EE^2$
$NE (Mcal\ kg^{-1}\ DM) = (2.2 + 0.136*GP_{24} + 0.057*CP + 0.149*EE)*2.2/ 14.64$
where:
OMD = organic matter digestibility in %.
ME = concentration of metabolizable energy in MJ kg^{-1} DM.
NE = concentration of net energy in Mcal kg^{-1} DM, then net energy unit converted to be MJ kg^{-1} DM.
GP_{24} = gas production in ml after incubation of 200 mg DM sample for 24 h.
CP = crude protein concentration of the sample (%DM)
CA = crude ash concentration of the sample (%DM)
EE = sample ether extract concentration (%DM).

In addition, short chain fatty acids (SCFA) were calculated from the following formula according to Getachew et al. (2002).
$SCFA (mmol\ 200\ mg^{-1}\ DM) = 0.0222*GP_{24} - 0.00425$
GP_{24} = gas production in ml after incubation of 200 mg sample DM for 24 h

Microbial protein (MP) was also calculated as 19.3 g microbial nitrogen per kg of OMD according to Czerkawski (1986).

Statistical analysis: The obtained data were analyzed by one way analysis of variance (ANOVA) and means were compared for the significance using Duncan's Multiple Range Test. Comparisons were considered significantly different if $P<0.05$. All analyses were performed using the SPSS Statistics 17.0 program (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Rumen fermentation, *in vitro* gas production and kinetics: Cumulative gas production as affected by substitution of ruminant diet with different rations of *H. gardnerianum* (HG) or *P. undulatum* (PU) is shown in Table 3. In addition, the predicted cumulative gas production profiles as defined by the model $p = a + b (1-e^{-ct})$ are presented in Figure 1. During the fermentation course, at most of incubation times, values of the produced gas by diets replaced with 25 and 50% of both plants were significantly higher ($P<0.05$) than those obtained by control diet (C) and the treatments substituted with the higher percentages 75 and 100% of HG or PU. The total gas produced by the treatment HG25% after 96 h of incubation significantly surpassed ($P<0.05$) all other treatments recording 49.91 ml 200 mg^{-1} DM, followed by 45.08 ml 200 mg^{-1} DM for the treatment PU25%. Moreover, there were similarities between the observed and the predicted volumes (from the exponential model) of gas production as concluded from Table 3 and Figure 1.

Kinetics of gas production obtained from the exponential model $p = a + b (1-e^{-ct})$ as a result of addition of HG or PU to the ruminant feed are shown in Table 3. The fermentation results revealed that the attained values by the treatments HG25, 50% and PU25% for the parameter (a) were 2.95, 2.64 and 2.67 ml 200 mg^{-1} DM, respectively. While, the figures for the parameter (b) were 52.45, 46.94 and 47.37 ml 200 mg^{-1} DM, respectively. All of these fermentation parameter values were significantly ($P<0.05$) greater than those obtained by the treatment C (a, 2.41 and b, 42.84 ml 200 mg^{-1} DM). On the contrary, when ruminant feed substituted with HG or PU in higher percentages 75 and 100%, values of gas fermentation parameters (a and b) were significantly lower ($P<0.05$) than those got by the treatment C. However, the obtained figures for the parameter (c) were in range 0.0179 – 0.0248 ml h^{-1} and showed there were no significant differences ($P<0.05$) between all treatments.

Table 3. Effect of replacement of ruminant diet with different levels of *H. gardnerianum* or *P. undulatum* on cumulative gas production (ml 200 mg^{-1} DM) and gas kinetics.

Treatment	Incubation time (hour)								Gas kinetic parameters		
	3	6	9	12	24	48	72	96	a (ml 200 mg^{-1} DM)	b (ml 200 mg^{-1} DM)	c (ml h^{-1})
C	2.87^{c}	6.82^{c}	10.22^{c}	15.38^{d}	21.14^{d}	26.07^{d}	36.27^{d}	40.73^{d}	2.41^{c}	42.84^{c}	0.0246^{a}
HG25%	3.52^{a}	8.36^{a}	12.52^{a}	18.84^{a}	25.91^{a}	31.94^{a}	44.44^{a}	49.91^{a}	2.95^{a}	52.45^{a}	0.0248^{a}
HG50%	3.15^{b}	7.48^{b}	11.21^{b}	16.86^{b}	23.18^{b}	28.58^{b}	39.77^{b}	44.66^{b}	2.64^{b}	46.94^{b}	0.0212^{a}
HG75%	2.74^{d}	6.51^{d}	9.76^{d}	14.69^{e}	20.19^{e}	24.90^{e}	34.64^{e}	38.90^{e}	2.30^{d}	40.88^{d}	0.0182^{a}
HG100%	1.70^{f}	4.03^{f}	6.05^{f}	9.10^{g}	12.51^{g}	15.42^{g}	21.45^{g}	24.09^{g}	1.43^{f}	25.32^{f}	0.0181^{a}
PU25%	3.18^{b}	7.55^{b}	11.31^{b}	17.02^{b}	23.40^{b}	28.85^{b}	40.14^{b}	45.08^{b}	2.67^{b}	47.37^{b}	0.0246^{a}
PU50%	2.95^{c}	7.0^{c}	10.49^{c}	15.78^{c}	21.69^{c}	26.74^{c}	37.21^{c}	41.79^{c}	2.47^{c}	43.92^{c}	0.0247^{a}
PU75%	2.74^{d}	6.51^{d}	9.76^{d}	14.68^{e}	20.19^{e}	24.89^{e}	34.63^{e}	38.89^{e}	2.30^{d}	40.87^{d}	0.0181^{a}
PU100%	2.37^{e}	5.62^{e}	8.43^{e}	12.68^{f}	17.44^{f}	21.50^{f}	29.91^{f}	33.59^{f}	1.98^{e}	35.30^{e}	0.0179^{a}
SEM	0.10	0.23	0.35	0.52	0.72	0.88	1.23	1.38	0.08	1.45	0.001

C = control feed diet (ordinary feed; 40% straw mixture, 40% grass and 20% concentrate); HG = *Hedychium gardnerianum*; PU = *Pittosporum undulatum*; a = gas production from the immediately soluble fraction (ml 200 mg^{-1} DM); b = gas production from the insoluble fraction (ml 200 mg^{-1} DM); c = gas production rate constant for the insoluble fraction (ml h^{-1}); SEM = Standard error of means.

$^{a, b, c, d, e, f, g}$; Mean values bearing the same superscript within a column are not significantly different at $P<0.05$.

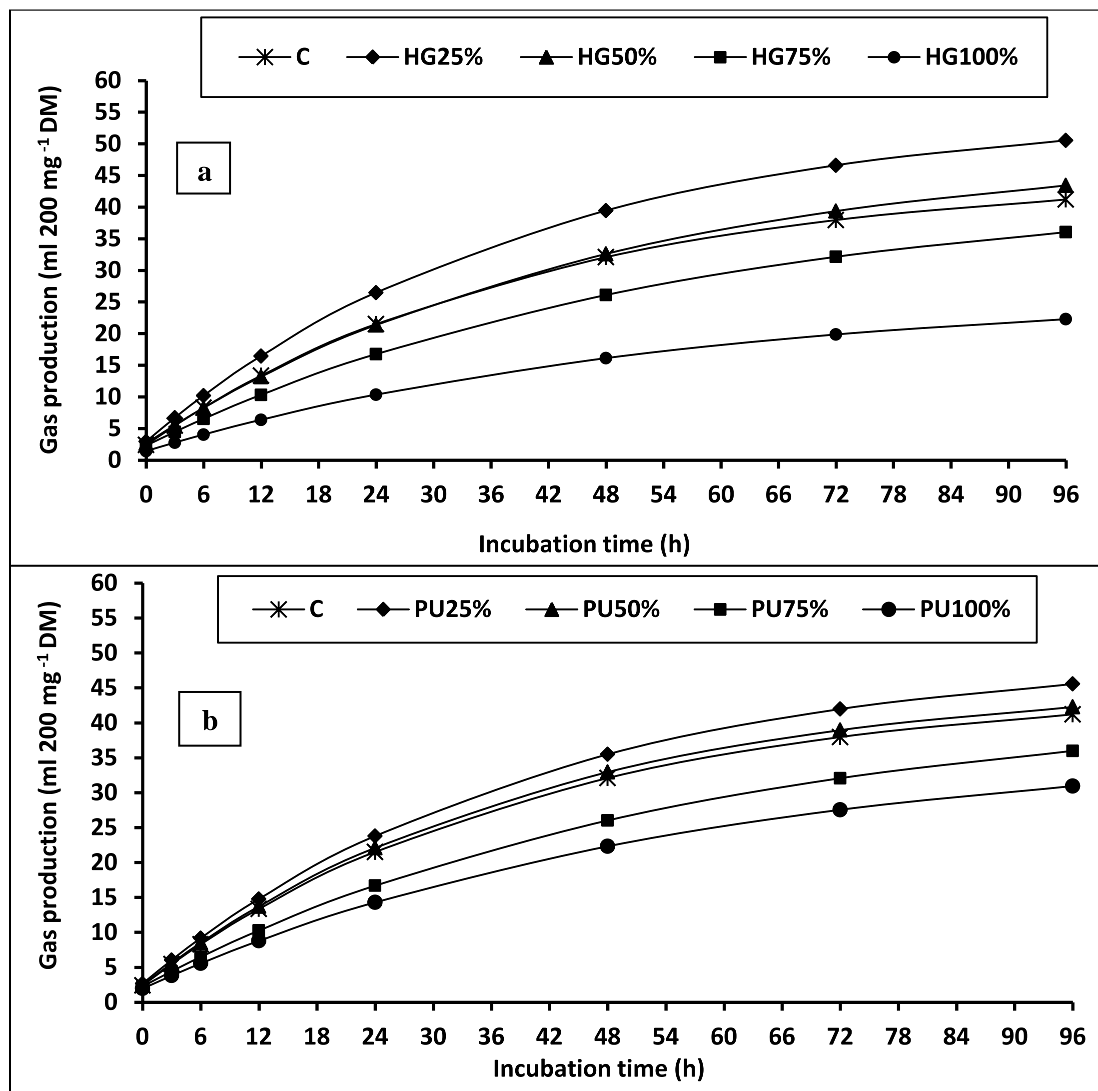

Fig.1. Cumulative gas production patterns through replacement of ruminant diet with different levels of *H. gardnerianum* (a) or *P. undulatum* (b) as defined *in vitro* by the model $p = a + b\ (1\text{-}e^{-ct})$.

Methane production: The results of methane production as presented in Table 4, showed the same trend of productivity that obtained in cumulative gas production. At the addition levels of 25 and 50% of HG or PU to the ruminant diets, the percentages of methane in the total gas mixture were 37.48, 31.62 and 33.87, 29.08%, respectively. These percentages appeared statistical significance (P<0.05) in relation to C treatment (27.53%). Again, when the addition levels of both plants were augmented to 75 and 100% an adverse effect was obtained for methane productivity. Accordingly, the obtained methane values were significantly lesser (P<0.05) than the C treatment, whereas the treatment HG100% recorded the least methane percentage (18.61%).

Microbial parameters: Total bacterial and protozoal counts as determined by the direct microscopic method and microbial protein (MP) production as calculated from organic matter digestibility are shown in Table 4. Regarding the total bacterial counts, the addition level 25% of HG and PU attained the highest bacterial figures, in that order 6.23 and 5.09 x 10^8 cell ml^{-1} which varied statistically (P<0.05) with bacterial numbers of the C treatment (4 x 10^8 cell ml^{-1}). However, the addition level 100% of both plants decreased bacterial counts significantly (P<0.05) to 1.92 and 2.67 x 10^8 cell ml^{-1}, respectively, as compared with the C treatment. Concerning the total protozoal counts, substitution of ruminant diet with HG or PU in levels of 25 and 50% significantly improved (P<0.05) the number of

Table 4. Effect of replacement of ruminant diet with different levels of *H. gardnerianum* or *P. undulatum* on methane (CH_4) and microbial protein (MP) production, pH, total counts of bacteria and protozoa *in vitro*.

Treatment	CH_4 (ml 200 mg^{-1} DM)	CH_4 (%)	pH	Bacteria x 10^8 (cell ml^{-1})	Protozoa x 10^5 (cell ml^{-1})	MP (g kg^{-1} OMD)
C	11.21[e]	27.53[e]	6.39[a]	4.0[cd]	3.73[b]	47.76[d]
HG25%	18.71[a]	37.48[a]	6.15[e]	6.23[a]	5.17[a]	52.20[a]
HG50%	14.12[c]	31.62[c]	6.27[bcd]	4.71[bc]	4.80[a]	48.79[c]
HG75%	9.99[f]	25.67[f]	6.31[abc]	3.08[de]	2.47[cd]	44.80[f]
HG100%	4.48[h]	18.61[h]	6.34[ab]	1.92[f]	2.0[d]	36.35[i]
PU25%	15.26[b]	33.87[b]	6.20[de]	5.09[b]	4.93[a]	49.50[b]
PU50%	12.15[d]	29.08[d]	6.22[cde]	4.30[bc]	4.70[a]	46.72[e]
PU75%	9.70[f]	24.95[f]	6.28[bcd]	3.26[de]	3.65[b]	44.17[g]
PU100%	7.45[g]	22.20[g]	6.30[abc]	2.67[ef]	3.0[c]	40.48[h]
SEM	0.79	1.09	0.02	0.26	0.22	0.90

C = control feed diet (ordinary feed; 40% straw mixture, 40% grass and 20% concentrate); HG = *Hedychium gardnerianum*; PU = *Pittosporum undulatum*; SEM = Standard error of means.
[a, b, c, d, e, f, g, h, i]; Mean values bearing the same superscript within a column are not significantly different at $P<0.05$.

protozoa (5.17, 4.80 and 4.93, 4.70 x 10^5 cell ml^{-1}, respectively) as referred to the C treatment (3.73 x 10^5 cell ml^{-1}). Similarly, numbers of protozoa significantly reduced ($P<0.05$) when HG and PU completely substituted the ruminant diet (2 and 3 x 10^5 cell ml^{-1}, respectively) as matched with the C treatment. For MP production, the highest significant ($P<0.05$) MP values were obtained when ruminant diet replaced with HG25, 50% and PU25% (52.20, 48.79 and 49.50 g kg^{-1} OMD, respectively) as compared with the C diet (47.76 g kg^{-1} OMD). Whereas, the least significant ($P<0.05$) MP value was obtained by the treatment HG100% (36.35 g kg^{-1} OMD).

pH measurements: A significant decrease ($P<0.05$) was noticed in the pH values from 7.0 in the buffered rumen to the range 6.15 – 6.39 in the fermented liquor of all treatments (Table 4). The highest pH value was recorded for the C treatment while the lowest one was recorded for HG25%. In general, it was observed that, the higher increase of addition level of both plants the higher of the pH values.

***In vitro* true dry matter digestibility, organic matter digestibility, short chain fatty acids, and energy contents:** Data presented in Table 5 reveals that, the *in vitro* true dry matter digestibility (IVTDMD) values for both HG and PU with the addition levels from 25 to 100% were in ranges 74.22 - 66.65% and 73.35 - 67.64%, respectively, whereas the C treatment got 70.88%.

The upcoming predicted values of different treatments were calculated from the amounts of gas produced at 24 h of incubation (Table 3) with the supplementary analysis of crude protein, fat and ash (Table 2). The predicted organic matter digestibility (OMD), short chain fatty acids (SCFA), metabolizable energy (ME,) and net energy (NE) as a result of addition of different levels of HG or PU to the ruminant diet are presented in Table 5. The OMD values varied significantly ($P<0.05$) among all treatments. The highest significant ($P<0.05$) OMD value was achieved by the treatment HG25% (43.27%) followed by the treatment PU at 25% (41.03%) whereas the C treatment attained OMD 39.59%. As well, when the ruminant diet replaced with HG or PU at levels 25 and 50%, the concentrations of SCFA were 0.567, 0.511 and 0.515, 0.477 mmol 200 mg^{-1} DM, respectively. This was significantly higher ($P<0.05$) than the C treatment (0.465 mmol 200 mg^{-1} DM). In contrast, when the ruminant diet received HG at addition level 100% the SCFA concentration was 0.273 mmol 200 mg^{-1} DM representing the least significant ($P<0.05$) value among all treatments. Also, it is evident from Table 5 that the ME and NE values showed higher significant differences ($P<0.05$) than the C treatment when ruminant feed received HG at level 25% (6.32 and 4.16 MJ kg^{-1} DM, respectively), followed by PU 25% (5.99 and 3.98 MJ kg^{-1} DM, respectively) and HG50% (5.91 and 3.91 MJ kg^{-1} DM, respectively).

Table 5. Effect of replacement of ruminant diet with different levels of *H. gardnerianum* or *P. undulatum* on *in vitro* true dry matter digestibility (IVTDMD), organic matter digestibility (OMD) short chain fatty acids (SCFA), metabolizable energy (ME) and net energy (NE).

Treatment	IVTDMD (%)	OMD (%)	SCFA (mmol 200 mg^{-1} DM)	ME (MJ Kg^{-1} DM)	NE (MJ Kg^{-1} DM)
C	70.88^{c}	39.59^{d}	0.465^{d}	5.74^{d}	3.78^{d}
HG25%	74.03^{a}	43.27^{a}	0.567^{a}	6.32^{a}	4.16^{a}
HG50%	72.22^{b}	40.45^{c}	0.511^{b}	5.91^{c}	3.91^{c}
HG75%	69.40^{d}	37.14^{f}	0.444^{e}	5.42^{f}	3.61^{e}
HG100%	66.65^{e}	30.14^{i}	0.273^{g}	4.36^{h}	2.95^{g}
PU25%	73.35^{a}	41.03^{b}	0.515^{b}	5.99^{b}	3.98^{b}
PU50%	72.23^{b}	38.73^{e}	0.477^{c}	5.67^{e}	3.79^{d}
PU75%	70.51^{c}	36.61^{g}	0.443^{e}	5.38^{f}	3.61^{e}
PU100%	67.64^{e}	33.56^{h}	0.382^{f}	4.94^{g}	3.35^{f}
SEM	0.47	0.75	0.02	0.11	0.07

C = control feed diet (ordinary feed; 40% straw mixture, 40% grass and 20% concentrate); HG = *Hedychium gardnerianum*; PU = *Pittosporum undulatum*; SEM= Standard error of means.
$^{a, b, c, d, e, f, g, h, i}$; Mean values bearing the same superscript within a column are not significantly different at $P<0.05$.

While, the treatment HG100% attained the least significant ($P<0.05$) energy values 4.36 and 2.95 MJ kg^{-1} DM, for ME and NE, respectively.

DISCUSSION

The current work was designed to assess the effect of substitution of ruminant feed with different levels (0, 25, 50, 75 and 100%) of *H. gardnerianum* (HG) or *P. undulatum* (PU) on *in vitro* gas production, CH_4 production and gas kinetics. In addition, to determine the nutrition value, energetic parameters, *in vitro* digestibility factors and microbial activities as affected by the addition of both plants.

***In vitro* gas and methane production:** In the present work, the obtained results demonstrated that, the addition of HG or PU in 25 and 50% to the ruminant diet, attained statistical variation ($P<0.05$) and greatly enhanced the net gas volumes by 22.54, 9.65% and 10.68, 2.60%, respectively as compared with the control (C). These findings may be attributed to the presence of the associative effect and/or the co-digestion effect within the ruminant diet as a result of addition of HG or PU. Blummel et al. (1997) suggested that the different nature of ingredients in the feed mixtures might cause asynchrony in the release of the nutrients, resulting in a different microbial biomass growth and activity which in turn might increase gas production. As well, the co-digestion has numeral benefits like; dilution of toxic compounds, improved balance of nutrients, synergistic effect of microorganisms and better gas yield (Hartmann and Ahring, 2005).

Getachew et al. (2004) and Maheri-Sis et al. (2007) stated that, gas production is positively correlated with NFC content of feeds, and negatively correlated with feed CP and NDF levels. Our results showed an opposite trend when HG and PU were added to the ruminant diet at levels 25 to 75%, the net gas volumes of HG were higher than PU, regardless, the NDF and NFC were 64.50 and 15.41%, respectively for HG vs. 43.84 and 40.70% for PU (Table 1). These results could be due to the high content of lignin (15.24% in PU vs. 7.03% in HG, Table 1) and slowly fermented carbohydrates found in PU which lead to late microbial colonization. It is known that, lignin is connected to cell wall polysaccharides and restricts their biodegradation by acting as a physical barrier in which lowering the bioavailability of the substrate to microbial enzymes (Jung and Fahey, 1983). These results were in one line with our data for gas production kinetics (Table 3). Also, these results might be occurred because, the narrower carbon : nitrogen (C/N ratio) in HG mixtures (30.26/1 – 42.09/1) comparing with PU mixtures (31.29/1 – 55.39/1) as displayed in Table 2. It is generally found that during anaerobic digestion microorganisms utilize carbon 25–30 times faster than nitrogen. Thus to meet this requirement, microbes need a C/N ratio around 25–30/1 in fermented

substrates (Bardiya and Gaur, 1997). The obtained results are generally in agreement with those obtained by Hamid et al. (2007) and Akinfemi et al. (2009) who attained higher gas production 67 vs. 64 and 47.98 vs. 41.61 ml 200 mg^{-1} DM when tested different feed materials enclosed NFC 15% vs. 67.5% and 6.63% vs. 13.10%, respectively. Whilst, Njidda and Nasiru (2010) obtained 66 vs. 62 ml 200 mg^{-1} DM when tested feed material contained NDF 58.67% vs. 33.31%, respectively.

In the present study, when addition levels of both plants augmented to 75 and 100% the net gas volumes decreased significantly (P<0.05) in relation to the control diet. The net gas volumes declined by 4.49, 40.85% and 4.52, 17.53% in case of ruminant diets substituted with HG 75, 100% and PU 75, 100%, respectively. These findings may be referred to the increase of secondary metabolites concentrations in feed mixtures (Babayemi et al., 2004). Besides, the C/N ratio of these feed mixtures became wider which consequently affected the microbial growth and activity.

In the current study, remarkable reduction in CH_4 production was monitored when the ruminant diet replaced with HG or PU in 75 and 100% (10.88, 60.04 and 13.47, 33.54%, respectively) as compared with the C treatment. This variation of CH_4 reduction among feed mixtures could be due to the width of C/N ratio and/or the differing presence of the inherent secondary metabolites. The secondary metabolites contents of *H. gardnerianum* and *P. undulatum* have been extensively studied by many of research groups. Medeiros et al. (2000), Medeiros et al. (2003), Arruda et al. (2012) and our group (un published data) found that the main active compounds present in *H. gardnerianum* are flavonoids, alkaloids, saponins, tannins, diterpenoids and some essential oil components. Whilst, the most vital compounds existing in *P. undulatum* are triterpenoids, saponins and polyphenolic compounds as reported by Medeiros et al. (2003), Mendes et al. (2011), Sadgrove and Jones (2013) and our group (un published data). These active compounds present in both plants thought to be responsible for CH_4 reduction and they have been found to be toxic for many of the rumen microbes, especially ciliate protozoa and methanogens. The aforementioned data are in a harmony with the reduction in total counts of bacteria and protozoa that observed in the present study at respective addition levels of both plants.

In a study conducted by Sallam et al. (2009) attained a grand depression of CH_4 production estimated by 90.3% relative to control when supplemented the ruminant diet with 150 µl of eucalyptus oil.

On the contrary, at lower addition levels of both plants, the amounts of released methane significantly increased (P<0.05) in relation to the C diet. CH_4 volumes augmented by 66.90, 25.96% and 36.13, 8.39% over the C, when HG or PU was added in 25 and 50%, respectively. The reason of that, at lower concentrations of both plants, the secondary metabolites were either not present in quantities high enough to alter the fermentation process or that rumen microbes may quickly metabolize and convert them to non-toxic compounds (Sandoval Castro1 et al., 2003). Generally, in most cases, feedstuffs that show high capacity for gas production are also observed to be synonymous for high methane production (Njidda and Nasiru, 2010), and that was in consentient with our observations.

Kinetics of gas production: The soluble fraction (a) represents the easily attachable part by ruminal microorganisms and leads to much gas production (Blummel and Becker, 1997). Also, the microbial ecosystem responds well to the increased supply of readily available carbohydrates and there is an increase in the total microbial populations, fermentation rate and VFA production, unfortunately this also leads to a drop in pH. This information confirms the results obtained through the current study. Values for (a), intercept were positive in the incubations of all treatments during this study and ranged from 1.43 to 2.95 ml 200 mg^{-1} DM (Table 3). As well, at lower levels of addition of both plants until 50%, the amounts of gas were significantly different (P<0.05) comparing with the C diet and HG emerged higher values than PU. These data suggested that a shorter lag phase (in case of HG) due to the rapid microbial colonization on the substrate which might be occurred in the early stage of incubation. On the other hand, several authors (Khazaal et al., 1993; Blummel and Becker, 1997) reported negative values with various substrates when using mathematical models to fit gas production kinetics. This is due to either a deviation from the exponential course of fermentation or delays in the onset of fermentation due to the slow microbial colonization. The gas volume at asymptote (b) describes the fermentation of the insoluble fraction. Likewise, the gas volume at asymptote (b) behaved a similar tend to (a) and laid in rang 25.32 – 52.45 ml 200 mg^{-1} DM. Moreover, values of HG mixtures were greater than PU values until addition level 75%. The gas volumes at asymptote have the advantage of predicting feed intake. Rate of gas production (c) expressed in ml h^{-1}, revealed no significant differences between all treatments (P<0.05). Gas rate constant values were over range 0.0179 - 0.0248 ml h^{-1}. The higher rate of gas production possibly influenced by carbohydrate fractions readily available to the microbial populations.

Microbial parameters: In the rumen ecosystem bacteria along with protozoa, are the predominant microbes and by mass account for 40-60% of total microbial matter in the rumen. Moreover, they digest about 70% to 80% of the

digestible dry matter in the rumen (Krause et al., 2003). Also, the symbiotic relation between methanogens and ciliate protozoa may generate up to 37% of rumen methane emission (Finlay et al., 1994).

In the present study, at low addition levels 25 and 50% of HG or PU to the ruminant diet, the total counts of bacteria and protozoa were significantly ($P<0.05$) greater than the C treatment. Total bacterial count enlarged by 55.75, 17.75% and 27.25, 7.5%, respectively. Whereas, the total protozoal count increased by 38.61, 28.69% and 32.17, 26.01%, respectively. This elevation in microbial growth and activity could be due to the presence of associative and/or co-digestion effects as discussed before. These results are consistent with the higher rates, volumes of gas and CH_4 production and the consequently higher production of VFA with these lower replacement levels of both plants. Contrariwise, with raising the levels of addition of HG or PU to 75 and 100%, numbers of bacteria and protozoa were markedly reduced in relation to the C diet. Bacterial figures fallen by 23, 52% and 18.5, 33.25%, respectively. Likewise, protozoal counts inhibited by 33.78, 46.38% and 2.14, 19.57%, respectively. This reduction in counts of both microbial groups could be referred to the width of C/N ratio within feed mixtures and/or the presence of antimicrobial secondary metabolites at inhibitory levels. In the current study, bacterial counts ranged between 1.92 to 6.23 x 10^8 cell ml^{-1} while, protozoal counts were in a range 2.0 – 5.17 x 10^5 cell ml^{-1}. Our results are in a harmony with the findings of López et al. (1999) who found that the supplementation of ruminant feed with 6.25 mmol of sodium fumarate decreased both bacterial and protozoal figures from 5.4 to 4.7 x 10^8 cell ml^{-1} and 4.4 to 3.8 x 10^3 cell ml^{-1}, respectively which lead to reduction in CH_4 emission. In another study conducted by Jayanegara and co-workers (2011) to investigate CH_4 emissions from, tropical plants when incubated in ruminal fluid *in vitro*. They obtained higher numbers for bacteria (2.5 – 5.8 x 10^9 cell ml^{-1}) and lower for protozoa (1.1 – 3.0 x 10^4 cell ml^{-1}) comparing with our results.

The results of this study indicated that the predicted microbial protein (MP) values were in agreement with above mentioned values of total counts of bacteria and protozoa. Substitution of ruminant feed with HG 25, 50% and PU 25% enhanced the MP production by 9.30, 2.16 and 3.64%, respectively in relation to the C feed. With elevating the addition levels of both plants above 50%, MP production values moderately decreased. These results are in agreement with the results of Czerkawski (1986) who found that a highly significant correlation between MP syntheses predicted from OMD in the rumen and that predicted from VFA production. Noteworthy, rumen microbes are the major source of protein for the ruminants (Moran, 2005).

pH measurements: In the current study, the pH values in all culture syringes were in range 6.15– 6.39 which indicates the microbial growth and the activity were normal. These data pointed out that the buffering capacity of this *in vitro* system was sufficient to maintain conditions within the expected range. A major consequence when ruminal pH falls below 6 is that fibre digestion declines dramatically (Russell and Wilson, 1996). In addition, Shriver et al. (1986) obtained an optimal pH of 6.7 for the digestion of a mixed forage-concentrate diet. However, a negative effect on fermentation parameters was only observed below pH 5.8 when ryegrass was incubated *in vitro* by de Veth and Kolver (2001), who also reported that with forage substrates DM digestibility and microbial protein synthesis were optimized at pH 6.35 and 6.13, respectively.

Digestibility factors (OMD and IVTDMD): In the current study, the OMD% values were in a range 30.14 – 43.27% while, IVTDMD% values ranged from 66.65 to 74.03%. This results are compatible with these obtained by Njidda and Nasiru (2010) who achieved average values 39 and 70% for OMD and IVDMD respectively, when tested the digestibility of some forages from semi-arid regions in Nigeria. Whereas, Elmenofy et al. (2012) reported higher values for OMD and IVDMD with averages 49 and 77.5%, respectively, when examined ensiled rice residues with some additives. Moreover, in the present work, OMD values exceeded the C treatment by 9.30, 2.17 and 3.64% for treatments HG 25, 50% and PU 25%, respectively. Whereas, IVTDMD records surpassed the C treatment by 4.44, 1.89% and 3.48, 1.90% for treatments HG 25, 50% and PU 25, 50%, respectively). In general, with augmentation of both plants addition levels resulted in poor *in vitro* digestibility. These data are in one line with the reduction of microbial activity that observed in the present study. This reduction might be due to the increase of fiber content and the secondary metabolites concentrations at higher addition levels of both plants (Van Soest et al., 1991; Reed et al., 1990).

Energy parameters: Short chain fatty acids (SCFAs) represent up to 80% of the ruminants daily energy requirements. In the present study, SCFA records ranged between 0.273 – 0.567 mmol 200 mg^{-1} DM. Additionally, SCFA values markedly improved by 2.58 – 21.94% as compared with the C diet when both plants added in 25 and 50%. The estimated SCFA values in the current study were lower when compared with the figures (0.96 – 1.21 mmol 200 mg^{-1} DM) obtained by Hamid et al. (2007) who tested the digestibility of some tropical feeds. On the other hand, lower SCFA concentrations were obtained by Jonathan et al. (2012) who studied the bioconversion of

some agricultural wastes to ruminant feed. These variations between the studies may be referred to the differences in the chemical composition between such substrates.

In the current work, metabolizable energy (ME) values were over the range 4.36 – 6.32 MJ Kg^{-1} DM. In addition, ME values recorded an augment above the control diet (2.96 – 10.10%) when both plants added to the ruminant diet at lower concentrations (till 50%). Concerning the net energy (NE) values were in a range 2.95 – 4.16 MJ kg^{-1} DM. Again, when the ruminant diet replaced with both plants (till 50%) the NE values revealed higher values than the C diet (0.26 – 10.05%). Furthermore, the ME and NE values that observed during the present study represent considerable energetic values as compared with selected feedstuffs commonly used in cattle feeding (NRC, 2001), such as; alfalfa (ME 8.20, NE 4.98 MJ kg^{-1} DM), almond hulls (ME 7.91, NE 4.77 MJ kg^{-1} DM), apple pomace (ME 7.78, NE 4.68 MJ kg^{-1} DM), barely silage (ME 8.49, NE 5.18 MJ kg^{-1} DM) and sorghum silage (ME 7.49, NE 4.64 MJ kg^{-1} DM).

CONCLUSION

The results of the current study demonstrated that *H. gardnerianum* and *P. undulatum* are promising and valuable alternative feed resources. Both plants can be incorporated partially, up to 50% in ruminant feed mixtures to replace conventional roughage sources without major problems and with value added income. Substitution of ruminant feed with plant material from these species in rations above 50% had a little inhibitory effect on total gas and volatile fatty acids production and on *in vitro* dry matter digestibility. Methane mitigation was also achieved. It seems important to elucidate the underlying mechanism for CH_4 reduction without affecting the efficiency of the digestibility.

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