Global Veterinaria 16 (3): 276-284, 2016
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2016.16.03.102186

Methane Concentration, Organic Matter Digestibility, Metabolisable Energy and Short Chain Fatty Acid Production of Morphological Fractions of Stinging Nettle (*Urtica simensis*) Measured Through an *In vitro* Gas Test

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Abstract: Gas production, methane concentration, organic matter digestibility, metabolisable energy and short chain fatty acid production of whole and morphological fractions (leaf, flower and stem) of stinging nettle (*Urtica simensis)* was evaluated through *in vitro* gas test. Stem showed highest ($p<0.05$) fibre (NDFom, ADFom and ADL) and organic matter (OM) content and the least was for leaf. Leaf contained highest ($p<0.05$) CP and the least was for stem. The total gas production at all incubation hours (3, 6, 12, 24, 48, 72 and 96h) for flower were significantly ($p<0.05$) higher than those of the other fractions followed by leaf and the least was for stem. Gas production due to the immediately soluble fraction (a=7.9) and the rate of constant of gas production(c=0.035) were higher ($p<0.05$) in flower. Whole forage showed higher gas production from the insoluble but degradable fraction (b=68.8) and the potential gas production (a+b=67.1). Predicted metabolisable energy (ME), organic matter digestibility (OMD) and short chain fatty acid (SCFA) of flower was significantly ($p<0.05$) higher than those of the other morphological fractions. Whole forage and stem fraction showed higher ($p<0.05$) methane (CH_4) concentration per total gas production at 24h of incubation. After 72h of incubation flower showed the highest ($p<0.05$) CH_4 concentration. Forages with high protein content are usually enhance digestibility through supplying fermentable protein and fibre contents are related to poor digestibility. *In vitro* gas production, ME and OMD were positively correlated (r^2=0.80, 0.39 and 0.62 respectively) with CP but negatively correlated (r^2= -0.21) with CH_4. Fibre components (NDFom and ADFom) were negatively correlated with ME (r^2=-0.75 and -0.64 respectively) and OMD (r^2=-0.88 and -0.79 respectively) but NDFom and ADFom were positively correlated (r^2=0.50 and 0.42 respectively) with CH_4 concentration. Total gas production after 96h of incubation was positively correlated (r^2=0.64) with SCFA. Generally, leaf and flower fractions had relatively higher gas production, ME, OMD, SCFA and CP values, which have potential to be used as supplement feed. But further animal feeding experiment is needed to verify this conclusion.

Key words: Morphological Fraction · Stinging Nettle · Chemical Composition · Gas Production

INTRODUCTION

Because stinging nettle is a nutrient rich herb with high protein, amino acids and mineral content [1- 5], the potential uses of stinging nettle as animal forage attract many researchers. Silage from treated stinging nettle provides more than 200g/kgDM protein and had 74% dry matter digestibility [6]. Stinging nettle haylage replaced grass silage in the diet of lactating cows and production level in terms of milk output were maintained [7] and stinging nettle also reduces the incidence of rumen acidosis [8].

The nutrient composition of feed is commonly determined by its chemical composition. However, this doesn't provide sufficient information to determine true nutritive value of feeds. The *in vitro* gas production system helps to better quantify nutrient utilization and its accuracy in describing digestibility in animals has been validated [9].

Chemical composition, in combination with *in vitro* gas production, organic matter digestibility (OMD) and metabolisable energy (ME) concentration are widely used to determine the potential nutritive value of forages. Some studies evaluated the forage and nutritive value of

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stinging nettle using *in vitro* gas production experiment and chemical analysis [2, 3, 5]. The chemical composition and digestibility of forages are influenced by several factors including proportions of plant morphological fractions. An *in vitro* gas test was therefore conducted on morphological fractions of stinging nettle (*Utrica simensis*) to determine its nutritive value because there is limited information.

MATERIALS AND METHODS

Study Site and Sample Collection: The study area is located in the southern Ethiopia (7°06'-9°38'N and 38°25'-35°53'E) with an elevation range of 1851-2759 masl. The annual rainfall range of the area is between 1000 and 1200 mm. The whole stinging nettle plant was randomly collected from naturally grown plants approximately when three fourth of the plant stand was flowered. Edible parts of stinging nettle including leaves, flowers, stem and whole forage (consisting of leaves, flowers and stems) were separately weighed fresh on the field then oven-dried to constant weight at 65 °C for 48h. The dried foliage samples were hammer-milled through a 1mm sieve and used to analyze crude protein (CP), neutral detergent fibre (NDFom), acid detergent fibre (ADFom), acid detergent lignin (ADL) and *in vitro* gas production.

Chemical Analysis: Crude protein (CP) were determined Using [10]. Nitrogen (N) content was determined by the Kjeldahl method and crude protein (CP) was calculated as N × 6.25. The NDFom and ADFom were determined according to [11], the NDFom was determined without amylase and sodium sulphite. Both NDFom and ADFom were expressed without residual ash.

***In vitro* Gas Production:** About 200 mg of dry sample (milled through a 1.0 mm sieve) was incubated *in vitro* with rumen fluid in a calibrated glass syringe of 100 ml in triplicate. Vaseline was applied to the pistons to ease movement and prevent escape of gas. The syringes were pre-warmed at 39°C before addition of 30 ml of buffer mixture and rumen liquor into each syringe. The syringes were shaken gently 30 min after the start of incubation and every hour for the first 10 h of incubation. Blanks with buffered rumen fluid without feed sample were also included in triplicate. All the syringes were incubated in a water bath maintained at 39°C. Gas production was recorded after 3, 6, 12, 24, 48, 72 and 96 h of incubation. The gas production characteristics were estimated by fitting the mean gas volumes to the exponential equation of Ørskov and McDonald [12]: $G = a + b\,(1\text{-}e^{-ct})$, where G is the gas production (ml/200mg OM) at time t, a is the intercept of the gas production curve, b is the extent of gas production, $a + b$ is the potential gas production (ml/200 mg OM) and c is the rate constant of gas production [13].

Organic matter digestibility (OMD) was calculated from the equation:

OMD (%) = OMD= 14.88 + 0.889GV+ 0.45 CP + 0.651ash [14].

where:

OMD = Organic matter digestibility at 48 hours.
CP = Crude protein content of feed samples
GV = Gas volume

Metabolisable energy (ME) was calculated from equation:

ME (KJ/gDM) =2.20+0.136GP+0.0057CP [14]

where:

GP = Gas production over 24rs of incubation
CP = Crude protein content of feed samples.

Short-chain fatty acids (SCFA) were estimated as:

SCFA = 0.0239GV-0.0601 [9].

where:

GV = Gas volume 24rs of incubation

Measurement of Methane Production: Methane production after 24 and 72 hours of incubation was measured using the procedure described by Fievez, Babayemi and Demeyer [15]. For measuring methane production at the end of incubations and after recording the final gas volume, the lower end of the syringe was connected to the lower end of another syringe containing 4.0 ml of NaOH (10 M). NaOH (10 M), which was then introduced from the latter into the incubated contents, thereby avoiding gas escape. Mixing of the contents with NaOH allowed absorption of CO_2, with the gas volume remaining in the syringe considered to be CH_4. Net

methane and gas productions were calculated by the differences of the methane and total gas in the test syringe and the corresponding blank. The methane concentration was calculated as Jayanegara *et al.* [16]:

Methane concentration = Net methane production/Net gas production.

Statistical Analysis: Statistical analyses were performed using the general linear model (GLM) procedure of the Statistical Analysis System [17]. Significance between individual means was identified using Fishers Least Significant Difference (LSD) and significance was declared at $P < 0.05$.

RESULTS

Crude Protein and Cell Wall Concentration: Crude protein (CP), organic matter (OM) and fibre components of morphological fractions of stinging nettle forage is presented in Table 1.

CP content ranged from 127 to 295g/kgDM. Leaf contained highest ($p < 0.05$) crude protein (CP) and the least was for stem. Stem showed highest ($p<0.05$) cell wall (NDFom, ADFom and ADL) content and the lowest was for leaf. Higher fibre and lower CP content was observed in stem and leaf showed the reverse of stem. The highest ($p<0.05$) organic matter (OM) content was for flower and the lowest was for leaf.

***In vitro Gas* Production:** The results of *in vitro* gas production of the morphological fractions of stinging nettle forage are presented in Table 2 and Figure 1. There was a significant difference ($p<0.05$) in total gas production among four different morphological fractions. The order of gas volume produced after 96h of incubation was as follows: flower > whole forage > leaf >stem. Flower showed the quick ($p<0.05$) gas production after 3h of fermentation followed by leaf and the least was for stem at this early incubation time. Gas production after 6, 12 and 24, 48 hours was highest for flower and least for stem, leaf and whole forage were in between flower and stem. However gas production after 48h of incubation was similar between whole forage and leaf fraction. The rapid gas production was observed between 12 and 48 hours of incubation (Figure 1). In general the highest ($p<0.05$) total gas production at 96h of incubation was recorded in flower and the least was for stem.

The *in vitro* gas production characteristics of the different morphological fraction of stinging nettle forage is presented in Table 2. Gas production due to the immediately soluble fraction (a) was higher ($p<0.05$) for flower. On the other hand, whole forage and stem showed lower and negative value of the immediately soluble fraction (a). Whole forage showed higher gas production from the insoluble but degradable fraction (b) and potential gas production (a+b). The rate of constant of gas production (c) was highest ($p<0.05$) in flower and least for whole forage and stem fraction. Leaf part exhibited intermediate rate of constant of gas production (c) between the two boundaries.

Methane Production, Metabolisable Energy, Organic Matter Digestibility and Short Chain Fatty Acid: Table 3 shows methane production and concentration, metabolisable energy (ME) and organic matter digestibility (OMD) of morphological fractions of stinging nettle forage. Variations in CH_4, ME and OMD were observed among morphological fractions. Whole forage and stem fraction showed higher ($p<0.05$) CH_4 concentration per total gas production at early time (24h) of incubation and the least was for flower and leaf frations. However after 72h of incubation it was reversed in which flower showed the highest ($p<0.05$) methane concentration and the least was for stem. Methane production ranged between 1 and 1.3ml/200mgDM after 24h of incubation and between 2.5 and 8.1 ml/200mgDM after 72h of incubation. Predicted metabolisable energy (ME) values of flower fraction was higher ($p<0.05$) than stem and whole forage. Similarly organic matter digestibility (OMD) was highest for flower and lowest for stem. The highest ($p<0.05$) SCFA was for flower and the lowest was for stem and whole forage.

Relationship of ME, OMD, CP, Fibre Content, Total Gas and Methane Production: Relationship among chemical composition, total gas and methane production, ME and OMD is presented in Table 4. Fibre fractions (ADFom and NDFom and ADL) were negatively correlated ($p<0.05$) with CP content, *in vitro* gas production, ME and OMD. On the other hand, CP content showed positive correlation ($p<0.05$) with *in vitro* gas production and OMD. A positive correlation ($p<0.05$) was observed among *in vitro* gas production, ME and OMD. The concentration of CH_4 was positively correlated ($p<0.05$) with fibre fractions but negatively correlated with CP.

Table 1: Chemical composition of morphological fractions of stinging nettle forage

	Leaf	Flower	Stem	Whole	SEM	P value
DM (g/kg)	176.2[b]	185.8[a]	151.8[d]	167.0[c]	2.3	<0.0001
CP (g/kgDM)	294.6[a]	227.4[c]	126.8[d]	254.6[b]	8.3	<0.0001
ADFom (g/kgDM)	133.9[d]	183.4[c]	363.6[a]	214.7[b]	2.1	<0.0001
NDFom (g/kgDM)	318.0[d]	330.3[c]	448.9[a]	393.6[b]	10.2	<0.0001
ADL (g/kgDM)	36.1[d]	75.5[b]	82.4[a]	42.9[c]	2.2	<0.0001
OM (g/kgDM)	760.7[d]	871.4[a]	838.2[b]	784.3[c]	3.9	<0.0001

*Means within a row with different superscripts differ significantly ($p < 0.05$).
CP= Crude protein, ADFom= Acid detergent fibre, NDFom= Neutral detergent fibre, ADL= Acid detergent lignin, OM= Organic matter, NFC= Non-fibre carbohydrates

Table 2: Gas production (and gas constants of the morphological fraction of stinging nettle forage incubated during the *in vitro* gas test

	Morphological Fraction					
Parameters	Flower	Leaf	Stem	Whole		
Incubation period (hrs)	Gas production (ml)				SEM	P-Value
24	41.8[a]	31.3[b]	18.7[c]	19.3[c]	1.282	0.0004
48	57.3[a]	46.0[b]	31.0[c]	43.0[b]	1.443	0.0012
72	60.3[a]	50.6[b]	35.0[c]	50.0[b]	1.080	0.0060
Gas production characteristics						
a	7.9[a]	4.4[b]	-0.23[c]	-1.7[c]	1.12	<0.0001
b	57.6[b]	50.7[c]	42.9[d]	68.8[a]	2.03	<0.0001
a+b	65.6[a]	55.1[b]	42.7[c]	67.1[a]	2.57	<0.0001
c	0.035[a]	0.033[ab]	0.024[b]	0.018[b]	0.002	<0.0001
L	-	-	0.5[b]	1.3[a]	0.47	0.0223

*Means within a row with different superscripts differ significantly ($p < 0.05$).
a = Gas production from the immediately soluble fraction (ml),
b = Gas production from the insoluble but degradable fraction (ml),
a + b = Potential gas production (ml),
c = The rate constant of gas production (fraction/h)
L= Lag time
SEM= Standard error of means

Table 3: Methane production and concentration, metabolisable energy and organic matter digestibility of morphological fractions of stinging nettle forage

	Methane (24h)		Methane (72h)				
Plant part	ml	ml/Total gas volume	ml	ml/Total gas volume	ME (MJ/kgDM)	OMD(%)	SCFA (µmol/gDM)
Flower	1.3	0.031[b]	8.1[a]	0.135[a]	8.0[a]	76.9[a]	0.9[a]
Leaf	1.3	0.031[b]	5.1[b]	0.101[b]	6.6[b]	70.5[b]	0.6[b]
Stem	1	0.053[a]	2.5[d]	0.071[d]	4.8[c]	49.1[d]	0.3[c]
Whole	1	0.068[a]	4.3[c]	0.086[c]	5.0[c]	65.9[c]	0.4[c]
SEM	0.204	0.0071	0.25	0.0042	0.17	1.18	0.034
P value	0.1189	0.0005	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

*Means within a column with different superscripts differ significantly ($p < 0.05$). CH_4= Methane ME= Metabolisable energy DM= Dry matter, OMD= Organic matter digestibility, SCFA=Short chain fatty acids, SEM= Standard error of means

Table 4: Correlation among nutrients, IVOMD, *in vitro* gas and methane production, calculated OMD and ME of morphological fractions of stinging nettle forage

	ADFom	NDFom	ADL	CP	TGP	CH_4	ME
ADFom	1.00						
NDFom	0.98**	1.00					
ADL	0.70**	0.57**	1.00				
CP	-0.95**	-0.89**	-0.86**	1.00			
TGP	-0.93**	-0.98**	-0.43	0.80**	1.00		
CH_4	0.42**	0.50**	0.03	-0.21	0.88**	1.00	
ME	-0.64**	-0.75**	0.07	0.39	0.81**	0.93**	1.00
OMD	-0.79**	-0.88**	-0.18	0.62**	0.97**	0.97**	0.89**

**Significant difference ($p<0.05$)CP= Crude protein, ADFom= Acid detergent fibre, NDFom= Neutral detergent fibre, ADL= Acid detergent lignin, TGP= Total gas production CH_4= Methane, ME= Metabolisable energy, OMD= Organic matter digestibility

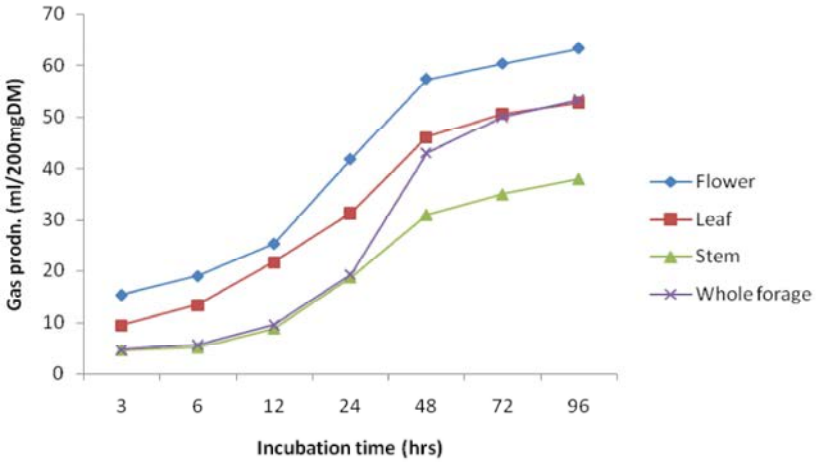

Fig. 1: *In vitro* gas production of morphological parts of stinging nettle forage

DISCUSSION

Chemical Composition: Stages of maturity, environmental conditions and morphological fractions of forages could affect chemical composition. Different morphological fractions of stinging nettle showed differences in chemical composition. The protein content of leaf obtained in the current study is comparable with reports of previous studies [18, 19] however [20] and Rafajlovska *et al.* [21] found lower values (25-26 and 18 -26 percent protein, respectively) but Rutto *et al.* [22] and Adhikari, Bajracharya and Shrestha [23] obtained higher (33%) leaf protein content. The protein content of stem found in the current study is in agreement with the results of Rafajlovska *et al.* [21].

The fibre content (NDFom, ADFom and ADL) of whole stinging nettle obtained in the current study is comparable with previous reports [2, 4]. On the other hand [5] reported higher fibre value but Wencelová *et al.* [3] reported lower fibre value than those of the current study. Leaf showed highest CP and lowest fibre (NDFom, ADFom and ADL) content and stem the reverse. Similar trend was reported by Debela and Tolera [24] and Leiber, Kunz and Kreuzer [25] on other leguminous trees and forages. The OM of whole forage obtained in the current study is comparable with the reports of Purcell *et al.,* Wencelová *et al.* and Pradhan, Manivannan and Tamang [2, 3, 18].

***In vitro* Gas Production:** Kinetics of gas production is dependent on the relative proportion of soluble, insoluble but degradable and un-degradable particles of the feed. A gas production profile allows analysis of data evaluation of substrate and media-related differences and ferment ability of soluble and slowly fermentable components of feeds [9]. The rapid gas production in the early stage of fermentation observed in flower and leaf fractions shows that they have higher content of rapidly fermentable soluble components. On the contrary slower gas production exhibited in whole forage and stem fractions might be due to low content of rapidly fermentable soluble components in these fractions. Moreover, as it is described by Tolera and Sundstøl [26] the higher insoluble feed components in these fractions (whole forage and stem) need to be hydrated and colonized by rumen micro-organisms before they can be fermented. Thus, before digestion of the insoluble feed components takings place, the microbial population has to multiply and colonize the substrate which results in increased rate of gas production in the early stages of incubation.

The gas production in leaf fraction was fast during early hours of incubation and consistent until 48h of incubation but declined then after. This could be due to the higher protein content of this fraction (Table 1). The incubation pattern of high protein feedstuffs, protein being part of the soluble fraction, is usually characterized by initial fast fermentation and reach maximum after 20h of incubation and after 46h of incubation protein content is likely fully fermented [27].

The observed differences in gas production parameters among morphological fractions indicate that there is a difference in rate and extent of fermentation characteristics. In stem and whole forage negative values of the readily degradable fraction (a) were recorded which agrees with earlier reports by Bezabih *et al.* [28] in grasses, woody species and forbs; and Debela *et al.* [29] for twigs and whole forage from Desmodium. Moreover [26] earlier reported that all morphological fractions of

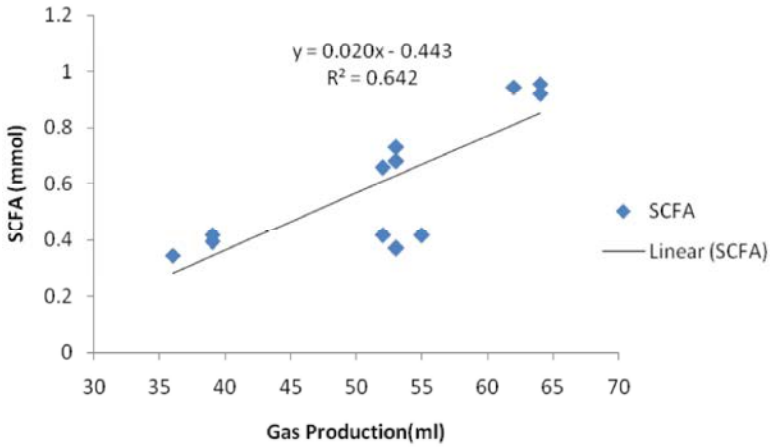

Fig. 2: Relationship between gas production and SCFA content

maize stover showed negative value of the readily degradable fraction (a). As it is explained by Blummel and Becker [30] the negative values could be due to differences in lag phase in the fermentation of insoluble feed components that lead to a deviation from the exponential curve of fermentation.

The higher value of gas production from slowly fermentable fraction (b) and the potential gas production (a+b) observed in whole forage and lower values for leaf and stem fractions could be associated with differences in protein content and fermentation pattern. The fermentation of protein reduces total gas production than carbohydrates [27].

The higher rate constant of gas production (c) observed in flower and leaf fractions could be associated with the content of soluble feed components in these fractions. Because the composition of the diet, its quality, deficiencies, excesses and availability of nutrients determine the speed of digestion. Generally, soluble components are fermented very rapidly, while less soluble substrates are attacked more slowly [31].

Organic Matter Digestibility, Metabolisable Energy and Methane Production: The OMD of flower fraction is comparable with some legume hays, such as alfalfa and common vetch [32]. The OMD of whole forage and leaf are comparable with some clover species but higher than some tropical legume hays including alfalfa hay [33, 34]. The stem fraction in this study showed relatively poor OMD. The predicted ME of flower fraction obtained in the current study is comparable with some alfalfa varieties [33] but less than the values obtained from some tropical legumes [32].

Amount of methane produced *in vitro* from whole forage in the current research is comparable with the report of Wencelová *et al*. [3], but higher than the result of Purcell *et al.* [2] and lower than the reports of Kulivand and Kafilzadeh [5]. Moreover, methane concentration recorded in the current study was lower compared with some forages of mid rift valley grasslands of Ethiopia [28], various tropical foliages [35] and some leguminous forages [36]. This could be due to the content of tannin and phenolic acids available in stinging nettle [20, 37] which could depress methanogenesis. The volume of methane produced increased with time of incubation. The higher absolute methane formation from flower and leaf over stem partition could be related to the higher total gas production observed in the former fractions. However, it is relevant to evaluated according to the proportion of methane to total gas produced at the particular fermentation hour than absolute methane formation. A low value of this proportion in flower fraction than stem after 24h of incubation time indicates a low methanogenic potential of flower and leaf than stem. Similar trends were observed in some tropical forage [28, 35].

The gas produced in the gas technique is the direct gas produced as a result of fermentation and the indirect gas produced from the buffering of SCFA. For roughages, when bicarbonate buffer is used, about 50 percent of the total gas is generated from buffering of the SCFA and the rest is evolved directly from fermentation. Hence gas production is proportional to SCFA content [9]. In the current study there was positive correlation (r=0.64) between amount of gas production and SCFA content (Figure 2). The highest SCFA content was in flower fraction and could be associated with the highest *in vitro* gas production of this fraction.

Relationship among ME, OMD, CP, Fibre Content, Total Gas and Methane Production: Forage materials with high protein content are usually able to supply fermentable protein which can be used to enhance digestibility. On the other hand fibre contents (NDFom and ADFom) are related to poor digestibility [38]. The observed negative correlation between fibre fractions (NDFom and ADFom) and *in vitro* gas production and the positive correlation between CP and *in vitro* gas production agrees with the trend reported by Kulivand and Kafilzadeh [5]. Similar relationships were also reported on tropical browses by Kaitho *et al.* [38]. The observed positive correlation of NDFom and ADFom with CH_4 concentration is in agreement with previous reports [5, 28]. Fermentation of NDFom usually increases methane production by shifting SCFA proportion towards acetate which produces more hydrogen [38]. In the present study, the ME content was consistent with OMD and gas production. A strong positive correlation among ME, OMD and *in vitro* gas production observed in the current study agrees with the findings of Evitayani *et al.* [39] and Karabulut *et al.* [32].

CONCLUSION

Morphological fractions varied in chemical composition, *in vitro* gas and methane production, predicted metabolisable energy (ME) and organic matter digestibility (OMD). The differences in contents of fibre components (NDFom and ADFom) and CP among different morphological fractions influenced digestibility and there by energy content and methane production. Hence fibre components had negatively influenced *in vitro* gas production, predicted metabolisable energy (ME) and organic matter digestibility (OMD), whereas the reverse happened with CP.

Based on the values obtained from the *in vitro* gas test in the current study, leaf and flower had higher ME, OMD, SCFA and CP content but relatively lower methane concentration at early stage of incubation than stem and whole forage. Hence both fractions could be used as supplements to low quality animal feeds. However, this has to be validated through animal evaluation.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the financial support of Development Partnerships in Higher Education (DelPHE) of British Council.

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