In vitro gas production, methane production and fermentation kinetics of concentrate diet containing incremental levels of sodium humate

Timothy Tertsegha IKYUME[1,2] (✉)
Olusiji Sunday SOWANDE[2]
Azeez Olanrewaju YUSUF[2]
Adebayo Olusoji ONI[3]
Peter Aniwe DELE[4]
Olalekan Toheed IBRAHIM[2]

Summary

The redox potentials of humic acids make it a veritable pathway to hydrogen consumption in the rumen that may be a strategy to mitigate methane production. This study assessed the fermentability indices and methane production of incremental levels of sodium humate by *in vitro* gas production technique. Five experimental diets containing sodium humate at 0, 5, 7.5 10 and 12.5 g/kg diet were formulated. Inoculum prepared was from rumen fluid of West African Dwarf (WAD) goats. Incubation period was 24 hours at 39°C. Fermentation kinetics, methane and rumen metabolites production were analysed using one-way analysis of variance as outlined in the GLM procedure of SAS. Results revealed a decrease ($p < 0.05$) in CH_4, VFA, acetate, propionate, butyrate, hydrogen consumed via CH_4/VFA pathway (HC), volume of gas from degradable fraction (A) and rate of gas constant (c) with addition of sodium humate in the diet up to 10 g/kg diet. There were increase ($p < 0.05$) in VFA, acetate, propionate, butyrate, HC, A and c at 12.5 g/kg diet humate inclusion. Also, hydrogen recovery (HR), metabolizeable energy (ME), adenosine triphosphate (ATP), microbial biomass, organic matter digestibility (OMD) and short chain fatty acids (SCFA) increased ($p < 0.05$) with incremental levels of sodium humate inclusion, but above 10 g/kg diet inclusion, ATP, MB, ME, OMD and SCFA were observed to decrease ($p < 0.05$). It was concluded that the impact of sodium humate in diet of goats is dose dependent and that supplementing the diet of WAD goats with sodium humate for up to 10 g/kg diet can effectively reduce methane production while also providing energy for rumen microbes and other metabolic activities of the animal.

Key words

[1] Department of Animal Production, Federal University of Agriculture, Makurdi, P.M.B. 2373, Nigeria

[2] Department of Animal Production and Health, Federal University of Agriculture, Abeokuta, P. M. B. 2240, Nigeria

[3] Department of Animal Nutrition, Federal University of Agriculture, Abeokuta, P. M. B. 2240, Nigeria

[4] Department of Pasture and Range Management, Federal University of Agriculture, Abeokuta, P. M. B. 2240, Nigeria

✉ Corresponding author: ikyumett@uam.edu.ng

Introduction

In ruminants, enteric methane (CH_4) is a major energy loss for the host animal. In addition, methane, a potent greenhouse gas, is contributing to climate change. Attempts by animal nutritionist are targeting at utilizing feed resources that will mitigate methane output from ruminants, while improving their overall performance. Feed additives such as garlic, humic substances, plant derivatives, and other natural antimicrobials (herbs and plant extracts) have been used as rumen modifiers in order to mitigate methane production (Busquet et al., 2005; Patra et al., 2006; Wanapat et al., 2008a; Wanapat et al., 2008b; Abarghuei et al., 2013; Ikyume et al., 2018; Sheng et al., 2018). However, literature on the use of humate as rumen modifiers in West African Dwarf (WAD) goats is still scanty.

Previous studies have shown that humic substances (HS) may have beneficial effects on livestock nutrition. Few studies on the use of humate or humic acid on feedlot performance, health and production in ruminants indicated a positive effect on the utilisation of carbohydrates and protein (Bell et al., 1997; Covington et al., 1997; Brown et al., 2007; Cusack, 2008). Inclusion of HS effectively reduced CH_4 production and increased substrate disappearance and the efficiency of microbial protein synthesis *in vitro* (Sheng et al., 2018). Terry et al. (2018) reported no effect of HS on CH_4 production in beef heifers under *in vivo* conditions. Similarly, comparable results were obtained for rumen variables in rams fed humic acid supplemented diets (Galip et al., 2010). The contrasting reports give room for the need to delve further into the use of humate as rumen modifier. In addition, there is paucity of information on the rumen modifier effect of sodium humate in ruminant nutrition with specific reference to WAD goats.

In vitro fermentation technique allows for estimation of methane and other fermentation kinetics due to the cumulative production of gases released during fermentation of the sample incubated in buffered rumen fluid (Pell and Schofield, 1993; Theodorou et al., 1994). In this milieu, this experiment evaluated the fermentation kinetics and metabolite production of concentrate diet containing incremental levels of sodium humate in WAD goats.

Materials and methods

Ingredient collection and formulation of diet

Sodium humate was purchased from a reputable company in china via aliexpres.com®. Maize, wheat offal, palm kernel cake, bone meal, mineral premix and salt were purchased from a feed shop in Abeokuta, Ogun State. The ingredients were milled into coarse form (not finely milled) and mixed together to form concentrate diet that was supplemented with 0, 5, 7.5, 10 and 12.5 g/kg diet of sodium humate, respectively (Table 1). About 200 mg of experimental diets were used as substrate for *in vitro* incubation.

In vitro gas production procedure and estimated values

The procedure of Babayemi and Bamikole (2006) was adopted for rumen fluid collection from three bucks that were fed a concentrate diet containing maize, palm kernel cake, wheat offal, and *Panicum maximum*. The rumen fluid collected were filtered with a 4-layer cheesecloth with the resulting ruminal fluid purged with deoxygenated CO_2 and put in a warm (39°C) thermo flask to be used with a buffer for preparation of inoculum. The buffer solution used was 9.8 $NaHCO_3$ + 2.77 $NaPO_4$ + 0.57 KCl + 0.47 NaCl + 2.16 $MgSO_4.7H_2O$ + 0.16 $CaCl_2.2H_2O$. The rumen fluid and the buffer were mixed together in ratio 1:2 (v/v). The procedure of Menke and Steingas (1998) was adopted for incubation of the samples with the use of a 100 ml calibrated transparent glass syringes fitted with silicon tube. Twenty replicates per treatment, each containing 200 mg of substrate (experimental diets), were put into pre-weighed Dacron fibre bags with a pore size of 50 μm and loaded in the syringes. Thirty milliliter of inoculum was drawn and dispensed into the calibrated transparent syringes containing the substrate under continuous CO_2 flushing. The piston of the syringes was pushed upwards to expel air bubbles. The silicon tubes on the syringes were properly clipped to prevent escape of gas before placement into the incubator at a temperature of 39°C. Gas volume was measured after 3, 6, 9, 12, 18, and 24 hr of incubation. Ten syringes containing only the inoculum were considered as the blank. The net gas productions of the samples were determined by correcting gas volumes for blanks.

Determination of methane

At post incubation period of 24 hr 4 ml of NaOH was introduced into six replicates, each per treatment, in the incubated samples to estimate methane production (Fievez et al. 2005). The introduction of NaOH was via silicon tube that was attached to the syringes. The differences between the volume of gas before and after introduction of NaOH was deduced to be the volume of methane.

Estimation of other fermentation kinetics

Metabolizable energy (ME) (MJ/kg diet) and organic matter digestibility (OMD) of dietary treatments during fermentation were calculated as established by Menke and Steingas (1998) while short chain fatty acids (SCFAs) produced during the period of fermentation were calculated as reported by Gatechew et al. (1989).

Total gas volume (GV) was expressed as ml/200 mg diet, CP and ash as g/kg diet, ME as MJ/kg diet and SCFA as μmol/g diet.

$$ME = 20 + 0.13GV - 0.057CP + 0.0029CF$$

$$OMD = 4.88 + 0.889GV + 0.45CP + 0.0651XA$$

$$SCFA = 0.0239GV - 0.0601$$

Where GV is Net gas production (ml/200 mg diet) during incubation

CP is Crude protein content of experimental diets

CF is Crude fibre content of experimental diets

XA is Ash content of experimental diets

Cumulative gas production data were fitted to non-linear exponential model as:

$$Y = A(1 - e^{-ct})$$

*a*CS

Agric. conspec. sci. Vol. 85 (2020) No. 2

Where Y is gas production at time 't', A is the volume of gas produced from degradable fraction with time (ml/200 mg diet), c is the gas production rate constant (h^{-1}) and t is the incubation lag time (h).

In vitro dry matter digestibility (IDMD)

The dry residues after the incubation were weighed and the digestibility was calculated as the percentage of the initial diet input. The formula used was:

IDMD = (Dry samples before incubation − dry samples after incubation) / (Dry samples before incubation) × 100

Volatile fatty acids and ammonia nitrogen

Total volatile fatty acids (VFAs) and proportions of acetate (A), propionate (P) and butyrate (B) were determined from incubated samples as described by Samuel et al. (1997). The samples were centrifuged at 3,000 xg for 10 min; they were allowed to settle and decanted. The decant was titrated with 0.1 M of sodium hydroxide (4/1000 gml^{-1} H_2O) solution each with 2 – 3 drops of phenophtaline (1/100 gml^{-1} ethanol) as the indicator. Determination of the various fractions was as enumerated below:

Acetate = (Titre Value × 0.1 × 0.06 × 100) / 5

Propionate = (Titre Value × 0.1 × 0.04 × 100) / 5

Butyrate = (Titre Value × 0.1 × 0.006 × 100) / 5

Total volatile fatty acids = (Titre Value × 0.1 × 0.09 × 100) / 5

Ammonia nitrogen was determined as described in AOAC (2005).

Hydrogen balance

Estimation of hydrogen balance was calculated according to formula given by Demeyer (1991) as follows:

Table 1. Gross composition of experimental diets

Parameter (kg)	Control	5HNa	7.5HNa	10HNa	12.25HNa
Maize offal	30	30	30	30	30
Wheat offal	34	34	34	34	34
Palm kernel cake	32	32	32	32	32
Bone meal	3	3	3	3	3
Vitamin premix	0.5	0.5	0.5	0.5	0.5
Salt	0.5	0.5	0.5	0.5	0.5
**HNa	-	0.5	0.75	1	1.25
Total	100	100	100	100	100
Determined analysis					
DM	88.00	88.00	88.00	88.00	89.00
CP% of DM	14.88	14.01	14.35	14.13	14.61
CF% of DM	9.50	9.50	10.00	9.00	10.00
Ash	5.00	5.40	5.45	6.00	6.50
EE% of DM	6.50	8.00	7.50	8.00	7.56
NDF% of DM	64.00	65.00	63.00	54.00	55.00
ADF% of DM	22.00	23.00	19.00	23.00	20.00
ADL% of DM	9.00	8.50	8.00	9.00	7.00

Control: 0 g/kg diet sodium humate inclusion
5HNa: 5 g/kg diet sodium humate inclusion
7.5HNa: 7.5 g/kg diet sodium humate inclusion
10HNa:10 g/kg diet sodium humate inclusion
12.5HNa: 12.5 g/kg diet sodium humate inclusion
 **HNa - used as a supplement and does not add up in diet formulation

Hydrogen recovery (HR):

$$HR(\%) = (4M + 2P + 2B) / (2A + P + 4B) \times 100$$

Hydrogen consumed via CH_4 / VFA $= 4M / (2P+2B)$

Where M is methane (mmol), P is propionate (mmol), B is butyrate (mmol) and A is acetate (mmol) produced during fermentation of dietary treatments.

Adenosine triphosphate (ATP)

ATP_{pr} is ATP produced during fermentation and it was estimated using concentrations of the VFA proportions by the following formula:

$$ATP_{pr} \text{ (mol)} = 2.5(A) + 2.75(P) + 3.5(B)$$
$$\text{(Owens and Goetsch, 1988)}$$

Microbial biomass

Microbial biomass was estimated as follows:

$$\text{Microbial mass (g)} = 10 \times ATP_{pr}$$

$$\text{(Owens and Goetsch, 1988)}$$

Chemical analysis

Proximate analysis of the substrate was done for dry matter, crude protein, crude fibre, ether extract and ash as described in AOAC (2005), while neutral detergent fibre, acid detergent fibre and acid detergent lignin were done as enunciated by Van Soest et al. (1991)

Statistical analyses

The *in vitro* kinetics, hydrogen efficiency, and production of methane, microbial biomass and adenosine triphosphate were analysed using one-way analysis of variance using the general linear models (GLM) procedures of SAS (2000). Significant differences among treatment means where applicable were seperated using Duncan multiple range test. Probability significance was declared at $P \leq 0.05$.

Results

Gas production parameters

The effect of incremental levels of sodium humate on *in vitro* gas production parameters of dietary treatments using ruminal fluid of WAD goats is shown in Table 2. Total gas volume (GV), ME, OMD and SCFA were the highest ($p < 0.05$) for the 10HNa treatment and reduced ($p < 0.05$) in control, 5HNa, 7.5HNa and 10.25HNa treatments, which had similar values of GV, ME, OMD and *in vitro* dry matter digestibility (IDMD) decreased ($p < 0.05$) at supplementation of sodium humate up to 10 g/kg diet. The highest ($p < 0.05$) similar values of IDMD were observed in control and 12.5HNa treatment (21.58% and 19.57%, respectively) and reduced ($p < 0.05$) in 5HNa, 7.5HNa and 10HNa treatments, which had the least similar values (15.95, 17.45 and 17.01%, respectively). Methane production was the highest ($p < 0.05$) in control group (9.40 ml/200 mg diet) and decreased ($p < 0.05$)

with increased humate supplementation. The highest ($p < 0.05$) lag time was comparable in control and 10HNa treatments and reduced ($p < 0.05$) in 5HNa, 7.5HNa and 12.5HNa treatments that had the least comparable values. Gas production fractional rate (c) was the highest in 10HNa group (0.012 ml/hr) that was similar to the control and 7.5HNa groups (0.0088 and 0.0081 ml/hr, respectively), and these differed significantly from 5HNa and 12.5HNa groups (0.00052 and 0.0014 ml/hr, respectively). The highest volume of gas produced from degradable fraction (A) was estimated for 5HNa group (33539.67 ml/200 mg diet), and this was similar to the control group (20256.93 ml/200 mg diet) but differed significantly ($p < 0.05$) from the 7.5HNa, 10HNa and 12.5HNa groups (5873.53, 3519.74 and 12474.86 ml/200 mg diet, respectively). The ratio of methane to gas volume was statistically similar ($p > 0.05$) for all treatment groups.

Rumen metabolites

Table 3 presents the rumen metabolites from *in vitro* fermentation of diet containing supplemental levels of sodium humates. Volatile fatty acids (VFA), acetate, propionate and butyrate from post-incubated samples had similar pattern in all the treatment groups. The highest ($p < 0.05$) comparable VFA, acetate, propionate and butyrate concentrations of post incubation samples were obtained in control and 12.5HNa treaments, while they were reduced in 5HNa, 7.5HNa and 10HNa treatments. Ammonia nitrogen and acetate to propionate ratio (A:P) were similar ($p > 0.05$) for the dietary treatments.

Fermentability indices

Fermentability indices as influenced by incremental levels of sodium humate in treatments *in vitro* gas production using ruminal fluid of WAD goats are shown in Table 4. The highest ($p < 0.05$) comparable amount of hydrogen recovery (HR) was observed in 10HNa and 12.5HNa treatments, while the least comparable amount of HR was found in control and 5HNa treatments. The amount of hydrogen consumed via the ratio of CH_4 and VFA (HC) decreased ($p < 0.05$) with humate inclusion The highest ($p < 0.05$) HC was observed in the control group (73.79), while the least comparable HC means were observed in 7.5HNa, 10HNa and 12.5HNa treatments. Production of adenosine triphosphate (ATP) and microbial biomass (MB) had the highest ($p < 0.05$) comparable amounts in 5HNa, 7.5HNa and 10HNa treatments, while the least ($p < 0.05$) comparable values were observed in control and 12.5HNa treatment.

Discussion

The volume of gas produced during fermentation reflects the products of the substrate fermentation to volatile fatty acids microbial biomass and neutralization of the VFA, and this is indication of the nutritional value of the substrate (Blummel and Becker, 1997). The reason for increased gas volume in 10 g/kg treatment may not have been inclusion of sodium humate in the diet, since all gas volumes were not affected by all other sodium humate supplemented treatment groups.

Dry matter digestibility is a measure of substrate disappearance. Decrease in the IDMD in the current study may have been due to the action of humic acid to prolong digestion period (Korniewicz

Table 2. *In vitro* gas production parameters from rumen of WAD goats fed diet containing supplemental levels of sodium humate

[1]Parameter	Control	5HNa	7.5HNa	10HNa	12.5HNa	SEM
GV(ml/200 mgDM)	21.54[b]	22.00[b]	22.80[b]	25.40[a]	22.90[b]	0.82
Methane (ml/200 mg DM)	9.40[a]	7.40[b]	5.00[c]	4.40[c]	4.20[c]	0.45
IDMD (%)	21.58[a]	15.95[b]	17.45[b]	17.01[b]	19.57[ab]	0.55
A (ml/200 mg DM)	20256.93[ab]	33539.67[a]	5873.53[b]	3519.74[b]	12474.86[b]	3146.74
c (ml/hr)	0.0088[a]	0.00052[c]	0.0081[ab]	0.012[a]	0.0014[bc]	0.0012
lag (hr)	-16.70[ab]	-19.10[b]	-17.13[b]	-13.80[a]	-18.83[b]	0.51
ME (MJ/kg DM)	4.24[b]	4.30[b]	4.40[b]	4.74[a]	4.41[b]	0.05
OMD(g/kg DM)	40.74[b]	41.16[b]	41.90[b]	44.20[a]	42.07[b]	0.31
SCFA (μmol/g DM)	0.45[b]	0.47[b]	0.48[b]	0.55[a]	0.49[b]	0.008
CH_4/GV (%)	19.22	17.30	11.58	8.16	9.48	2.20

[a,b,c] Means with different superscript along the same row differ significantly ($p < 0.05$)

[1] GV - gas volume, IDMD- *in vitro* dry matter digestibility, A - rate of gas production from soluble fraction, c - gas production constant, Lag - fermentation time, ME - metabolizeable energy, OMD - organic matter digestibility, SCFA - short chain fatty acid, CH_4/GV - methane-gas volume ratio

Table 3. Rumen metabolites of concentrate diet containing incremental levels of sodium humate from *in vitro* fermentation

Parameter	Control	5HNa	7.5HNa	10HNa	12.5HNa	SEM
NH3-N (g/100 ml)	40.26	30.06	43.09	39.69	37.99	2.05
VFA (Mm/100mol)	1.75[ab]	1.68[b]	1.57[b]	1.69[b]	1.92[a]	0.04
Acetate (Mm/100 mol)	1.17[ab]	1.12[b]	1.05[b]	1.10[b]	1.28[a]	0.02
Propionate (Mm/100 mol)	0.77[ab]	0.75[b]	0.70[b]	0.75[b]	0.85[a]	0.02
Butyrate (Mm/100 mol)	0.12[ab]	0.11[b]	0.10[b]	0.11[b]	0.13[a]	0.002
A:P	1.51	1.50	1.50	1.47	1.50	0.007

[a,b] Means with different superscript along the same row differ significantly ($p < 0.05$)

Table 4. Fermentability indices of concentrate diet containing incremental levels of sodium humates from *in vitro* fermentation

Parameter	Control	5HNa	7.5HNa	10HNa	12.5HNa	SEM
HR %	8.39[c]	10.64[c]	14.07[b]	16.81[ab]	19.14[a]	1.10
HC	73.79[a]	52.62[b]	34.39[c]	32.34[c]	36.66[c]	4.37
ATP (mol)	5.51[bc]	5.78[ab]	6.17[a]	5.84[ab]	5.06[c]	0.12
MB (g)	55.06[bc]	57.77[ab]	61.70[a]	58.39[ab]	50.56[c]	1.22

[a,b,c] Means with different superscript along the same row differ significantly ($p < 0.05$)

HR-hydrogen recovery; HC - hydrogen consumed via CH_4/VFA; ATP - adenosine tryphosphate; MB - microbial biomass

et al., 1999; Suchy et al., 1999), thereby suggesting it will take a longer time for the substrate to disappear. This implies that substrate disappearance with humate supplementation may increase if more time for incubation was given. This was further affirmed by increase in the lag time observed in this study with sodium humate supplementation. Increased lag time could result in low feed intake by the animal. The observed decrease in IDMD did not however affect the production of ATP, microbial biomass and short chain fatty acids.

The decrease in methane production in this study is consistent with the work of Sheng et al. (2018) who observed a reduction in methane production in *in vitro* conditions when humic substance was included for up to 30 g/kg diet. McMurphy et al. (2011) has reported that humic substances (HS) could exhibit a high affinity for nitrogen (N), and this property is considered to enhance rumen microbial biomass, reduce nitrogen excretion and methane (CH_4) release to the environment. The mechanism of lowering methane production may be adjured to be because of the redox capacity of humic substances that is attributed to the variety of functional structures, such as quinone, phenolic, hydroxyl, nitrogen and sulphur-containing molecules (Aeschbacher et al., 2010). This capacity makes humic substances including humates to act as electron acceptors for a wide variety of microorganisms capable of extracellular electron transfer, with methanogens inclusive (Martinez et al., 2013), and is evident in the decrease in the hydrogen consumed (HC) via the methane/VFA pathway in this study. The implication is that more of the hydrogen produced during the process of fermentation would have undergone a redox reaction with the humates instead of the methanogens. These redox potentials have been shown to lower methane production in soil microorganisms (Tan et al., 2018).

The rate of gas produced from degradable fraction (A) of the substrate decreased at higher concentration of sodium humate in the diet. Crude protein availability in the rumen is reported to be directly correlated with the rate of gas produced from degradable fraction (A) of the substrate (Akinfemi et al., 2009). Summarily, the crude protein available in the diets with sodium humates may not have been available for degradation in the rumen. This is because sodium humate reduces proteobacteria in the rumen (Mitsumori et al., 2002). The relatively low fractional rate of gas production (c) as affected by humate addition in this study is attributed to lower protein degradation in the rumen. Dele (2012) reported that higher values of fractional rate of gas production were attributed to the amount of nitrogen that was available for degradation under *in vitro* analysis. Thus, the lower fractional rate of gas production in some of the sodium humate groups may be indication that fewer nutrients were available for rumen microorganisms (Getachew et al., 2004). The non-significant difference observed for NH_3-N in this study is not consistent with reports of Bell et al. (1997) and Sheng et al. (2018). These differences could be attributed to the type of humate used for the various studies as well as source of rumen fluid.

Volatile fatty acids (VFA) as well as the various proportion of the VFA (acetate, propionate and butyrate) decreased at low-to-relatively high doses (5, 7.5 and 10 g/kg diet) of sodium humate used in this study compared to 12.5 g/kg diet. The addition of 12.5 g/kg diet did not affect the production of VFA, acetate, propionate and butyrate as compared to the control. The comparable means

between the control group and the 12.5HNa group is consistent with report of Terry et al. (2018) who did not observe significant differences in VFA and its proportions when beef heifers were fed a barley silage-based diet containing increasing concentrations of humic substance. Higher production of volatile fatty acids in the dietary treatment containing 12.5 g/kg diet of sodium humate means more energy available to the animal for production.

Fermentability indices such as hydrogen, microbial biomass and adenosine triphosphate (ATP) are indication of substrate appreciation. The increase in the rate of H_2 recovery (HR) with incremental levels of sodium humate inclusion could be attributed to less energy available. Energy available in form of ATP for the microbes involved in dehydrogenase reactions releasing H_2 was observed to reduce in 12.5HNa with a corresponding rise in HR in the same treatment group. It is worthy to note that while incremental levels of sodium humate addition did not decrease H_2 recovery (HR), its (HC) consumption was decreased via methane/volatile fatty acids. The mechanism of reduction in H_2 consumption is explained by the redox properties of humic substances that are hydrogen scavengers. The increase in microbial biomass in the sodium humate group up to 10HNa with a subsequent decrease in 12.5HNa group might be attributed to more hydrogen intake by the microbes. Less amount of HC in this study imply more hydrogen would have been available for the microbes. In addition, Leng (2014) has reported that with less crude protein from the diet for microbes, they will resort to use the non-protein-nitrogen source (NPN source) which is bound to increase microbial growth efficiency and then, hydrogen intake. In this report as HC decreased, MB increased. But this is also dose dependent as above supplementation of 10 g/kg diet, microbial biomass decreased.

Conclusion

The supplementation of sodium humate in the diet of WAD goats effectively reduced methane production while also improving the fermentability of the diets. However, these effects were dose dependent as supplementing sodium humate above 10 g/kg diet reduced energy and microbial biomass in the rumen. Sodium humate can therefore be included in the diet of WAD goats for up to 10 g/kg diet.

Conflict of interest

The authors declare that there was no conflict of interest in the course of the research work.

References

Abarghuei M. J., Rouzbehan Y., Salem A. Z. M., Zamiri M. J. (2013). Nutrient digestion, ruminal fermentation and performance of dairy cows fed pomegranate peel extract. Livest Sci 157 (3): 452–461. doi: 10.1016/j.livsci.2013.09.007.

Aeschbacher M., Sander M., Schwarzenbach R. P. (2010). Novel electrochemical approach to assess the redox properties of humic substances. Envtl Sci Tech 44: 87–93. doi: 10.1021/es902627p

Akinfemi A., Adesanya A. O., Aya, V. E. (2009). Use of an in vitro gas production technique to evaluate some Nigerian feedstuffs. American-Eurasian J Sci Res 4 (4): 240-245

Association of Official Analytical Chemists (AOAC). 2005. Official methods of analysis. 18th ed. AOAC Int., Gaithersburg (MD)

Babayemi O. J., Bamikole M. A. (2006). Effects of Tephrosia canidida leaf and its mixtures with guinea grass on in vitro fermentation changes as feed for ruminants in Nigeria. Parkistan J Nutr 486 (5): 14-18. doi: 10.3923/pjn.2006.14.18

Bell K.W., Byers F. M., Greene L. W. (1997). Humate modification of fermentation of forage/grain diets in continuous culture. J Anim Sci 75: 269 (abstract)

Blummel M., Becker K. (1997). The degradability characteristics of fifty-four roughages and rouphages neutral detergent fiber as described in vitro gas production and their relationship to voluntary feed intake. Brit J Nutri 77: 757-768

Brown M. S., Lawrence T. E., Ponce C. H., Pulikanti R., Smith S. R., Mitchell C. S. (2007). Effects of a humate product on growth performance, carcass merit, and tissue and serum mineral composition of individually fed steers. J Anim Sci 85: 357-361

Busquet M., Calsamiglia S., Ferret A., Carro M. D., Kamel C. (2005). Effects of cinnamaldehyde and garlic oil on rumen microbial fermentation in a dual flow continuous culture. J Dairy Sci 88(7): 2508-2516. doi: 10.3168/jds.S0022-0302(05)72928-3

Cone J. W., Valk H. (1997). Rumen fermentation kinetics of grass measured in sacco and with the gas production technique. In: Proceeding of the XVIII international grassland congress, Winnipeg, Manitoba, Canada, pp. 35-36

Covington B. R., Ramsey S., Greene L. W., Byers F. M. (1997). Effects of humate on feedlot performance and carcass characteristics in feedlot lambs. J Anim Sci 75: 270 (Abstract).

Cusack P. M. V. (2008). Effects of a dietary complex of humic and fulvic acids on the health and production of feed lot cattle destined for the Australian domestic market. Austra Vet J 86: 46-49. doi: 10.1111/j.1751-0813.2007.00242.x

Dele P. A. (2012). Evaluation of dry matter yield and Nutritive Quality of Forage Hay and Silage Produced from three Grasses Fertilized with Animal Manures. PhD. Federal University of Agriculture, Abeokuta, College of Animal Science and Livestock Production, Abeokuta.

Demeyer D. I. (1991). Quantitative aspects of microbial metabolism in the rumen and hindgut. In: J. P. Jouany (Editor). Rumen Microbial Metabolism of Ruminant Digestion. INRA Editions, Paris, France, pp. 217-237

Fievez V. O., Babayemi D., Demeyer D. (2005). Estimation of direct and indirect gas production in Syringes: A tool to estimate short chain fatty acid production that require minmal laboratory facilities. Anim Feed Sci Tech 123: 197-210

Galip N., Polat U., Biricik H. (2010). Effects of supplemental humicacid on ruminal fermentation and blood variables in rams, Italian J Anim Sci 9: 390-393. doi: 10.4081/ijas.2010.e74

Getachew G., De Peters E. J., Robinson P. H. (2004). *In vitro* gas production provides effective method for assessing ruminant feeds. California Agric 58(1): 154-58

Getachew G., Makkar H. P. S., Becker K. (1989). Stoichiometric relationship between short chain fatty acid and *in vitro* gas production in presence and absence of polyethylene glycol for tannin containing browses. In: EAAP Satellite Symposium, Gas Production: Fermentation Kinetics for feed Evaluation and to asses Microbia Activity. Wageningen, pp. 46-47.

Ikyume T. T., Sowande O. S., Dele P. A., Yusuf A. O., Monday S., Egunjobi O. K., Fatoba O. (2018). *In vitro* fermentation and rumen microbial count of West African Dwarf goats fed garlic (*A. sativum*) powder. Bull Anim Hlth Prod Afri 66 (3): 491-499

Korniewicz D., Korniewicz A., Dobrzanski Z., Kolacz R., Czarnik-Matusewicz H., Paleczek B. (1999). Efficiency of mineral-fat supplement Humobentofet in boar and gilt nutrition. Ann Anim Sci – Rozc Nauk Zoot 26:275-289

Leng R. A. (2014). Interactions between microbial consortia in biofilms: a paradigm shift in rumen microbial ecology and enteric methane mitigation. Anim Prod Sci 54: 519-543. doi: 10.1071/AN13381

Martinez C. M., Alvarez L. H., Celis L. B., Cervantes F. J. (2013). Humus-reducing microorganisms and their valuable contribution in environmental processes. Appl Microbiol Biotechnol 97: 10293–10308. doi: 10.1007/ s00253-013-5350-7

McMurphy C. P., Duff G. C., Sanders S. R., Cuneo S. P., Chýrase N. K. (2011). Effects of supplementing humates on rumen fermentation in Holstein steers. S Afri J Anim Sci 41(2): 134-140. doi: 10.4314/sajas.v41i2.71017

Menke K. H., Steingass H. (1988). Estimation of the energetic feed value obtained by chemical analysis and *in vitro* gas production using rumen fluid. Anim Res Dev 28: 7–55

Mitsumori M., Ajisaka N., Tajima K., Kajikawa H., Kurihara N. (2002). Detection of roteobacteria from the rumen by PCR using methanotroph-specific primers. Lett. Appld Microbio 35: 251–255. doi: 10.1046/j.1472-765X.2002.01172

Owens F. N., Goetsch A. I. (1988). Ruminal Fermentation. In: The Ruminal Animals, Digestive Physiology and Nutrition (Church D. C. ed). Prentice Hall, New Jersey, pp. 145-171.

Patra A. K., Kamra D. N., Agarwal N. (2006). Effect of spices on rumen fermentation, methanogenesis and protozoa counts in *in vitro* gas production test. Int Cong Ser 1293: 176-179. doi: 10.1016/j.ics.2006.01.025

Pell A. N., Schofield P. (1993). Computerised monitoring of gas production to measure forage digestion. J Dairy Sci 76: 1063-1073. doi: 10.3168/jds.S0022-0302(93)77435-4

Samuel M., Sagathewan S., Thomas J., Mathen G. (1997). An HPLC method of estimation of volatile fatty acids of ruminal fluid. Indian J Anim Sci67: 805-811

SAS Institute (2000). SAS/E-miner 3.0 program. SAS Institute Inc., Cary, North Carolina.

Sheng P., Ribeiro Jr G. O., Wang, Y., McAllister T. A. (2018). Humic substances supplementation reduces ruminal methane production and increases the efficiency of microbial protein synthesis *in vitro*. J. Anim. Sci 95: 300. doi: 10.2527/asasann.2017.613

Suchý P., Herzig I., Písaříková B. (1999). The use of sorbents on the basis of humic acids to reduce ammonia levels in stable environment. Vet Med Czech 44: 331-338

Tan W., Jia Y., Huang C., Zhang H., Li D., Zhao X., Wang G., Jiang, j., Xi B. (2018). Increased suppression of methane production by humic substances in response to warming in anoxic environments. J. Environ. Mgt 206: 602–606. doi: 10.1016/j. jenvman.2017.11.012

Terry S. A., Ribeiro G., Gruninger R. J., Hunerberg M., Ping S., Chaves A. V., Burlet J., Beauchemin K. A., McAllister T. A. (2018). Effect of humic substances on rumen fermentation, nutrient digestibility, methane emissions, and rumen microbiota in beef heifers. J Anim Sci 96 (9): 1-15. doi: 10.1093/jas/sky265

Theodorou M. k., Williams B. A., Dhanoa M. S., McAllen A. B., France J. (1994). A simple gas production method using pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim Feed Sci Tech 48: 185-197. doi: 10.1016/0377-8401(94)90171-6

Van Soest P. J., Robertson J. B., Lewis B.A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 74: 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2

Wanapat M., Cherdthong A., Pakdee P., Wanapat S. (2008b). Manipulation of rumen ecology by dietary lemongrass (*Cymbopogon citrates* Stapf) powder supplementation. J Anim Sci 86: 3497–3503. doi: 10.2527/jas.2008-0885

Wanapat M., Khejorsart P., Pakdee P., Wanapat S. (2008a). Effect of supplementation of Garlic powder on rumen ecology and digestibility of nutrients in ruminants. J Sci Food Agric 88: 2231–2237. doi: 10.1002/jsfa.3333

---

aCS85_20

aCS

Agric. conspec. sci. Vol. 85 (2020) No. 2