Wayamba Journal of Animal Science – ISSN: 2012-578X; P347 - P355 2012
First Submited July 20, 2012; Number 1343455597

EFFICACY OF *garlic, eucalyptus and neem* POWDERS ON RUMEN MODULATION, METHANOGENESIS AND GAS PRODUCTION KINETICS IN WHEAT STRAW BASED DIET EVALUATED *in vitro*

Kumar S.S., Navneet G., Mehta M., Mohini M., Pandey P., Shete S. and Brisketu K.
Nutrition Biotechnology Lab, Dairy Cattle Nutrition Division, National Dairy Research Institute, Karnal, 132001-Haryana, (INDIA)

Corresponding Author: sirohisk@gmail.com

The present study was carried out with the objectives to evaluate the effect of different plant parts on methane production as rumen fermentation modulators and gas production kinetics by using *in vitro* gas production technique. Garlic (*Allium sativum*, T_1) Eucalyptus (*Eucalyptus globules*, T_2) and Neem (*Azadiracta indica*, T_3) plant part powders were used for the evaluation for their inhibitory action on methane production, rumen fermentation and gas production kinetics on three type of wheat straw based diets i.e. high fiber diet (HFD), medium fiber diet (MFD) and low fiber diet (LFD). Methane was estimated by Gas Chromatography and results indicated that the maximum (51.96%) methane reduction was found on the supplementation of garlic powder in HFD as compared to that of control diet. Partition factor, microbial biomass, acetate to propionate ratio and gas production (b) was increased due to the addition of garlic powder. Garlic powder also showed the significant ($P \leq 0.05$) reduction in protozoa number in all type of diets.

KEY WORDS: Plants powder, Methane, IVDMD, *In vitro* gas production technique, Gas production kinetics.

Atmospheric concentration of green house gases (GHGs) increases due to the increasing of global human population, unlimited use of fossil fuels, urbanization etc. Other then human activities, ruminant livestock have been recognized as a major contributor to greenhouse gases (Steinfeld et al., 2006). Livestock account for mainly 80% of all emissions from the agricultural sector. In ruminants, some end product of rumen fermentation process such as methane and ammonia are energetically wasteful and harmful and also cause ecological problems because methane is a potent greenhouse gas (NRC 1992); the global warming potential of methane is 21-times that of CO_2 over 100 years (UNFCCC, 2007). Methane emission from ruminants reduced the efficiency of nutrient utilization and about 2 to 12% energy was wasted in terms of gross energy intake (Jonson and Jonson, 1995).

Many ionophores**,** antibiotics have been used to improve the rumen fermentation (Nagaraja, 1995), improving the some end product (propionate) and decreasing the total amount of methane (Stanier and Davies, 1981). However, the use of antibiotics as a feed additive in ruminants has been banned in the European Union, Since, January 2006, due to the risk of its residue in animal products (e.g.: milk and meat) and its subsequent effects on human health (Russell and Houlihan, 2003). Therefore, safe and cost effective new alternatives are needed to maintain efficient animal production systems. Plant extract and plant parts contained high concentration of secondary compounds and can be used as a safe means of ruminal fermentation modulators (Teferedegne 2000). The present experiment was planned to see the effect of three herbal plant parts i.e. Garlic (*Allium sativum*) Eucalyptus (*Eucalyptus globules*) and Neem (*Azadiracta indica*) on rumen fermentation and methane reduction under *in vitro* conditions.

MATERIALS AND METHODS

Procedure of plant powder preparation

The tested herbal plant parts i.e., Garlic bulbs (*Allium sativum*, T_1) was purchased from local vegetable market and Eucalyptus leaves (*Eucalyptus globules*, T_2) and Neem (*Azadiracta indica*, T_3) leaves were manually collected from National Dairy Research Institute, Karnal, India. The plants materials were dried over night in hot air oven at 70^0C and ground in mills to pass a 1 mm sieve and store in an air tight container. Finely grounded plant parts were used as a supplement.

Preparation of diets

To evaluate the effect of T_1, T_2 and T_3 three diets were prepared by taking different roughage and concentrate ratio i.e. high fiber diet (HFD, 60R:40C), medium fiber diet (MFD, 50R:50C) and low fiber diet (LFD, 40R:60C) and milled to pass through 1 mm sieve and used as substrate. The roughage part composed of wheat straw and the concentrate part composed of maize (33%), groundnut cake (21%), mustard cake (12%), wheat bran (20%), de-oiled rice bran (11%), mineral mixture (2%) and salt (1%) respectively.

Treatments and experimental design

2% (DM basis) of each treatment were added to the different wheat straw based HFD, MFD and LFD diets. All the treatment combinations were arranged in 4 × 3 factorial designs with three replicates. Set was also incubated devoid of substrate with and without powders which served as blanks for particular treatment and values were corrected for different parameters with these blanks.

Preparation of inoculums and *in vitro* gas production

Rumen liquor was collected after manual mixing of rumen contents from a fistulated mature male buffalo (*Bubalus bubalis*) maintained on a standard diet (60 parts roughage: 40 parts concentrate) before morning feeding into a pre-warmed insulated flask and brought into the laboratory. The rumen liquor filtered through four layers of muslin cloth and then the required amount of filtered rumen liquor used as a source of inoculum. The incubation medium was prepared as per previously described method (Menke and Steingass 1988). Treatments was added in 100 ml glass syringe containing 200±10 mg of milled (1mm) three type wheat straw based diets. The 30 ml incubation medium was dispensed anaerobically in each syringe. Plungers of syringes applied with petroleum jelly for smooth movement and stop any leakage. Syringes were closed using clamps and were incubated at 39 ± 0.5^0C for 24 h.

Estimation of methane production by gas chromatography

Methane content in fermentation gas was determined by gas chromatography (GC) as described by (Sirohi *et. al.,* 2012) using Nucon-5765 gas chromatograph. For methane estimation, each gas sample (250µl) was manually injected using Hamilton airtight syringe. Methane content in sample was calculated by external calibration, using a certified gases mixture with 50% CH_4 and 50% CO_2 (Spantech calibration gas, Surrey, England). The peak of methane gas was identified on the basis of retention time of standard methane gas and the response factor obtained was used to calculate methane percentage in the gas sample. The methane produced from substrate during 24 hour incubation was compared for the blank values. The volume of methane produced was calculated as follows:

Methane production (ml) = Total gas produced (ml) × % methane in the sample.

Rumen fermentation parameters

TVFA concentration (mM/100 ml) in the supernatant was estimated according to prescribed method (Barnet and Reid, 1957). For the estimation of IVFA, 1 ml of the supernatant was treated with 25% meta-phosphoric (4 ml) and kept for 3-4 h at ambient temperature (Erwin et al., 1961). Thereafter, IVFA was estimated using gas chromatograph according to the prescribed method (Sirohi *et. al.*, 2012). Sample (2 μl) was injected through the injection port using Hamilton syringe (10 μl). Individual VFAs of the samples were identified on the basis of their retention time and their concentration (mmol) and calculated by comparing the retention time as well as the peak area of standards after deducting the corresponding blank values. For the estimation of ammonia nitrogen, the supernatant of each syringe including that of

blank was used for NH_3-N estimation. Supernatant (5 ml) was mixed with 1 N NaOH (12 ml) and steam passed on this using KEL PLUS - N analyzer (Pelican, India) and the NH_3 evolved was collected in boric acid solution having mixed indicator and titrated against N /100 H_2SO_4.

Partitioning factor and microbial biomass yield

The PF is calculated as the ratio of substrate truly degraded dry matter *in vitro* (mg) to the volume of gas (ml) produced by it. Substrate provides important information about partitioning of fermentation products. The MBM yield was calculated by using the degradability of substrate and gas volume and stoichiometrical factor (Blummel et al., 1997).

Microbial mass (mg) = Substrate truly degraded - (gas volume × stoichiometrical factor)

Where the stoichiometrical factor used was 2.25.

Protozoa counting

For protozoal count, one milliliter of the fermentation fluid was diluted with 1 ml of formalin (18.5% formaldehyde) and 3-4 drops of brilliant green and then incubated for 24 hours at room temperature. The stained protozoa were diluted (if needed) and counted by haemocytometer as per the prescribed method (Dehority, 1984).

***In vitro* true DM degradability**

To estimate true DM degradability of feed sample of each syringe containing residues after incubation was estimated as per the prescribed method (Van Soest *et al.,* 1991).

Proximate analyses and Cell wall constituents

The proximate analysis of substrate was carried out as per the methods of AOAC (1995). The cell wall constituents of substrates were determined according to described method (Van Soest *et al.,* 1991).

Gas production kinetics

The total gas production kinetics was carried out in different plant powders incubated as per procedure mentioned above for different intervals i.e. 0, 1, 2, 3, 6 9, 12, 24, 36, 48, 60, 72 and 96 h. The potential gas production and rate of gas production was calculated by fitting the modified equation (Orskov *et. al.,* 1979)

Regression model = Orskow without lag Equation, F=b*(1-exp (-c*x))

Statistical analysis

Experimental data of different parameters were analyzed in factorial arrangement of randomized block design with three replicates for analysis of variance as per Snedecor and Cochran, 1968 in OPSTAT statistical software developed by Chaudhry Chran Singh Haryana Agriculture University, Hissar, Haryana, India. When the overall *F*-test was significant, differences between means and the control were declared significant at $P \leq 0.05$ using the Fisher's-Least-Significant-Difference (Critical Difference).

RESULTS

The physical and chemical composition of all the three wheat straw based diets are shown in Table 1. Results of different herbal plant powder supplementation on *in vitro* rumen fermentation pattern, *in vitro* dry matter digestibility and methanogenesis are presented in table 2 and 3.

Effect of plant powder on pH was significant for all three diets in all treatments. Maximum pH was found in T_2 (7.27) in HFD and minimum was in T_2 (7.05) in LFD. Maximum (0.16) unit variation as compared to control was found in T_2 in case of HFD. Digestible dry matter (mg) portion from 200 mg of feed dry matter was maximum increased i.e. 25.8, 19.5 and 25.7% on the supplementation of T_2 in case of HFD, MFD and LFD respectively (Table 2). While on adding T_1, IVDMD did not affect significantly.

In present experiment, both partition factor (PF) and microbial biomass production (MBM in mg) were increased with the supplementation of T_1 (table 2). The highest increase in PF was 49.68, 28.57 and 41.17% and highest increase in MBM (mg) was 77.73, 91.13 and 120% as compared to control in HFD, MFD and LFD, respectively. A reduction in methane production (mM/gDDM) was seen on the supplementation of T_1, whereas, it decreased methane 51.96, 21.86 and 20.21% in HFD, MFD and LFD diets, respectively. T_2 and T_3 did not show any significant reduction in methane production. A little variation in

TVFA concentration was observed in all type of diets. Results indicated that TVFA concentration either approximately same as control or decreased. Maximum reduction of TVFA concentration (21.13%) was observed in HFD on supplementation with T_2.

Table 1: Physical and chemical composition of wheat straw based diets used as substrate

Ingredient of diets							
Diets	**g/kg on DM basis**						
	Wheat straw	**Concentrate**					
HFD	600	400					
MFD	500	500					
LFD	400	600					
Ingredient of concentrate							
Particulars	**g/kg on DM basis**						
Maize	330						
Ground nut cake	210						
Mustard cake	120						
Wheat bran	200						
Deoiled rice bran	110						
Mineral mixture	20						
Salt	10						
Chemical constituents of diets (g/kg on DM basis)							
Diets	**OM**	**CP**	**EE**	**NDF**	**ADF**	**HC**	**TA**
HFD (60R:40C)	867.6	108.6	23.4	623.1	372.0	251.1	132.4
MFD (50R:50C)	878.4	125.3	30.4	604.5	329.5	275.0	121.6
LFD (40R:60C)	875.6	142.7	34.8	538.7	298.7	240.0	124.4

HFD=High fiber diet, MFD=Medium fiber diet, LFD=Low fiber diet, OM=Organic matter, CP=Crude protein, EE=Ether extract, NDF=Neutral detergent fiber, ADF=Acid detergent fiber, HC=Hemicelluloses, TA=Total Ash

Table 2: IVDMD and other parameters affected by supplementation of different plant powders

Diets	Treatments	Parameters					
		pH	IVDMD%	PF	MBM (mg)	CH_4 (mM/gDM)	Protozoa x10^5/ ml
HFD (60R:40C)	Control	7.11	53.3	3.22	32.56	3.31	0.82
	T_1	7.22	52.1	4.82	57.87	1.59	0.40
	T_2	7.27	60.6	3.79	55.50	2.87	0.46
	T_3	7.10	59.3	2.98	30.56	3.66	0.62
MFD (50R:50C)	Control	7.14	64.6	3.01	32.37	3.75	0.77
	T_1	7.10	66.5	3.87	61.87	2.93	0.37
	T_2	7.26	68.7	3.49	53.43	3.67	0.48
	T_3	7.10	62.1	3.10	36.70	2.80	0.75
LFD (40R:60C)	Control	7.09	56.1	3.06	28.93	2.77	1.53
	T_1	7.07	59.6	4.32	63.75	2.21	0.50
	T_2	7.05	61.6	3.66	53.50	2.82	1.40
	T_3	7.10	58.6	3.07	35.25	3.85	0.80
SEM	Diet	0.015	0.72	N.S.	N.S.	0.11	0.97
	Treatment	0.017	0.83	0.19	3.23	0.13	N.S
	D*T	0.030	1.44	N.S.	N.S.	0.23	0.19

T_1=Garlic powder, T_2=Eucalyptus powder, T_3=Neem powder, HFD=High fiber diet, MFD=Medium fiber diet, LFD=Low fiber diet, IVDMD=*In vitro* dry matter digestibility, PF=Partition factor, MBM=Microbial biomass, CH_4=Methane, SEM=standard error of means

Almost a decreasing trend in acetate concentration was observed in all type of diets except in LFD, whereas acetate concentration was increased (5.33%) with supplementation of T_3. Maximum reduction (22.95%) in acetate concentration was noticed in HFD supplemented with T_2. Same trend observed in case of propionate concentration. Results indicated that concentration of propionate was increased only with supplementation of T_3 in all type of diets and maximum increase (15.46%) propionate concentration was observed in HFD. Slight change in A/P ratio was observed in MFD and LFD in all treatments, while in case of HFD, it was increased. A non significant ($P \leq 0.05$) reduction in ammonia nitrogen was observed in current experiment. NH_3-N concentration decreased with supplementation of all treatments in HFD, MFD and LFD and the maximum reduction (13.15%) was found in LFD with T_2. In present study, a significant ($P \leq 0.05$) reduction in protozoal numbers was observed with the supplementation of T_1 and maximum reduction i.e. 67.32, 51.94 and 51.00% was found in LFD, MFD and HFD, respectively.

Results related to gas kinetics of wheat straw based HFD, MFD and LFD due to plant powder supplementation presented in table 4. It was observed from the results that gas production (b) values were increased due to supplementation of all powders in all diets. The increase in b values was highest in T_1 and T_3 in case of HFD, MFD and LFD and in case of T_1, the increase was highest (38.81%) in HFD, while, in case of T_3 increase was (18.66%) in MFD as compared to control. Similarly, gas production rate constant (c) also increased in almost all the treatments in comparison to control diet with high, medium and low fiber wheat straw based diets.

DISCUSSION

Methane is a potent green house gas, 15 to 20% of the global production of methane by ruminants (Crutzen et al., 1986) and its production in rumen nutritionally wasteful

process which represents 2 to 15% feed energy loss (Moss, 1993).

Table 3: Volatile fatty acid and ammonia nitrogen affected by supplementation of different plant powders

Diets	Treatments	Parameters					
		TVFA (mM/100ml)	Acetate (mM/100ml)	Propionate (mM/100ml)	Butyrate (mM/100ml)	A:P	NH_3-N (mg/100 ml)
HFD (60R:40C)	Control	6.31	5.01	0.97	0.32	5.16	20.53
	T_1	5.25	4.35	0.67	0.22	6.47	18.10
	T_2	4.85	3.86	0.75	0.26	5.29	18.01
	T_3	6.10	4.66	1.12	0.31	4.17	20.25
MFD (50R:50C)	Control	6.15	4.80	1.03	0.31	4.71	22.68
	T_1	6.30	4.83	1.15	0.31	4.22	21.65
	T_2	5.90	4.55	1.07	0.35	4.28	22.61
	T_3	6.10	4.62	1.13	0.34	4.09	23.61
LFD (40R:60C)	Control	6.43	4.69	1.33	0.40	3.64	23.42
	T_1	6.11	4.52	1.27	0.32	3.55	21.93
	T_2	6.15	4.54	1.26	0.35	3.66	20.34
	T_3	6.83	4.94	1.43	0.45	3.45	20.53
SEM	Diet	0.14	N.S	0.04	0.01	0.14	0.35
	Treatment	0.16	0.12	0.04	0.01	0.17	0.41
	D*T	N.S	N.S	N.S	N.S	0.29	0.71

T_1=Garlic powder, T_2=Eucalyptus powder, T_3=Neem powder, HFD=High fiber diet, MFD=Medium fiber diet, LFD=Low fiber diet, TVFA=Total Volatile Fatty Acids, A: P= Acetate to Propionate Ratio, NH_3-N= Ammonia Nitrogen, SEM=standard error of means

Table 4: Kinetics of gas production as affected by supplementation of different plant powders

	Treatment	Gas kinetics parameters		
		b	C	R^2
HFD (60R:40C)	Control	32.90	0.05	0.995
	T_1	45.67	0.08	0.996
	T_2	35.41	0.06	0.994
	T_3	37.04	0.07	0.998
MFD (50R:50C)	Control	36.64	0.06	0.998
	T_1	40.44	0.09	0.995
	T_2	39.41	0.08	0.982
	T_3	43.48	0.08	0.996
LFD (40R:60C)	Control	42.91	0.09	0.993
	T_1	46.65	0.10	0.993
	T_2	42.91	0.07	0.979
	T_3	49.15	0.08	0.988

HFD=High fiber diet, MFD=Medium fiber diet, LFD=Low fiber diet, b=Potential gas production (ml), c=Gas Production Rate Constant (ml/h), R^2 =Regression Coefficient

Due to the safe, cost effective and easy availability, plant parts or plants extract are used for the rumen manipulation. Methane production (mM/gDDM) either not affected due to T_2 or T_3 supplementation or decrease by T_1 (Garlic powder) supplementation. This tendency to reduced methane formation by T_1 (Garlic powder) was not surprising because the organo-sulphur compounds of garlic oil could inhibit rumen methanogenic archaea by inhibiting the enzyme HMG-CoA reductase (Busquet *et al.* 2005a, b, 2006; Kamel and Greathead 2008; Kamra *et al.* 2012).

In current study, IVDMD is significantly affected due to the supplementation of herbal plant powders and significant increase was seen on T_2 inclusion. This finding is in accordance with Busquet *et al.* (2005a), Yang *et al.* (2007) and Kongmun *et al.* (2010). The PF of the diets is an index of microbial biomass synthesis efficiency (Blummel *et al.* 1997) and the diet formulation to achieve higher PF would mean aiming for higher microbial biomass synthesis *in vivo* (Blummel *et al.* 2003). In present experiment, we observed that, both PF and MBM was increased on inclusion with herbal plant parts and highest increased was seen on T_1 addition. Decrease in TVFA, acetate concentration (mM/100 ml) slight change in propionate concentration and decrease in A:P ratio was observed in supplementation with herbal plant powder, which is in accordance with previous study done by Patra et al. 2006 and Hristov et al., 2003. Protozoa concentration was significantly ($P \leq 0.05$) reduced in all diets on supplementation with T_1 (Garlic powder). Hence, it is clearly demonstrated that active ingredients present in garlic powder could affect on growth of protozoa as also reported by Busquet *et al.,* (2005a), Busquet *et al.,* (2005b) and Kongmun *et al.* (2010).

CONCLUSION

In present study, it was concluded that 2% garlic powder emerged out as a promising candidate of supplementation in wheat straw based diet than the other herbal plant powders in HFD, MFD and LFD without any adverse affect for effective mitigation of methane under *in vitro* condition.

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