IJAS Iranian Journal of Applied Animal Science

Ruminal Methane Emission, Microbial Population and Fermentation Characteristics in Sheep as Affected by *Malva sylvestris* Leaf Extract: *in vitro* Study

Research Article

S. Khamoshi[1], F. Kafilzadeh[1], H. Jahani-Azizabadi[2*] and V. Naseri[1]

[1] Department of Animal Science, Faculty of Agriculture, Razi University, Kermanshah, Iran
[2] Department of Animal Science, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran

Received on: 27 Sep 2016
Revised on: 21 Jan 2017
Accepted on: 31 Jan 2017
Online Published on: Jun 2017

*Correspondence E-mail: ho.jahani@uok.ac.ir

Online version is available on: www.ijas.ir

ABSTRACT

The objective of this study was to investigate *in vitro* effect of *Malva sylvestris* leaf extract (at 0, 25, 50 and 100 µL/30 mL of medium) on sheep ruminal cellulolytic and total viable bacteria growth, protozoa populations, methane production, neutral detergent fiber degradability (NDFD) and fermentation efficiency of oat hay. The addition of *Malva sylvestris* leaf extract at 25, 50 and 100 µL led to a linear increase (P<0.01) *in vitro* truly degraded dry matter (INTDDM), NDFD and partitioning factor (PF) of oat hay and decrease (P<0.01) methane emission after 24 hours of incubation. The addition of *Malva sylvestris* leaf extract resulted in a decrease (P<0.01) in potential of gas production at 25 and 50 µL and increase in lag time (0.95, 1.01 and 1.13 h relative to 0.61 h), constant rate of gas production (*b*) and gas produced at half-life (*c*) at 25, 50 and 100 µL. The addition of this extract decreased a number of total protozoa and *Entodinium*, *Isotrichae*, *Diplodinium* and *Ophryoscolex* species (P<0.01). The number of total and cellulolytic bacteria was not influenced by the addition of *Malva sylvestris* leaf extract. The result of this study demonstrated that *Malva sylvestris* extract had some potential for improving the rumen fermentation.

KEY WORDS cellulolytic bacteria, kinetics, partitioning factor, protozoa, rumen.

INTRODUCTION

With increase in the probability of transmissible antibiotic resistance to human, the use of growth-promoter antibiotics in animal production systems has been banned in most developed countries (European Union, 2003). The removal of antibiotics has led the scientists' interest on investigation for the alternatives (Busquet *et al.* 2005; Iason, 2005; Durmic *et al.* 2014). Medicinal plants and their extracts as natural alternative to antibiotics showed the potential of manipulating the rumen fermentation to improve the utilization of nutrients (Busquet *et al.* 2005; Kim *et al.* 2013; Kim *et al.* 2015). The bioactive constituents of these compounds are secondary metabolites that are produced by plants to protect themselves against plant diseases and insects (Wallace, 2004). *Malva sylvestris* usually known as common Mallow is native to Asia, Europe, and north Africa and have been used as a medicinal plant since a long time ago. In the Mediterranean region, *Malva sylvestris* has a long history of use as food and potent drug in traditional and ethno veterinary medicines because of its anti-inflammatory, antioxidant, anti-complementary, anticancer and skin tissue integrity characteristics (Gasparetto *et al.* 2011). The whole aerial parts of *Malva sylvestris* or its leaves have been administrated to ruminant to treat colic, blocked rumen, mastitis, young calves' diarrhea, respiration problem and reproductive disorder (Gonzalez *et al.* 2010; Idolo *et al.* 2010).

Enteric methane arising due to fermentation of feeds in the rumen not only is a great source of energy loss but contributes substantially to the greenhouse gas emissions. In the recent years with increase horse farm in Kermanshah province the request for oat grain increased. Therefore, increase in oat cultivation resulted to enhance in oat hay and straw production that mostly have been used in the sheep nutrition. Thus, like an evaluation of chemical composition and nutritive values of feeds, methane production potential of each feed should be determined (Patra *et al*. 2015). The effect of *Malva sylvestris* on ruminant colic and improve the blocked rumen, may be because of its effect on rumen microbial activity. Therefore, the objective of the present experiment was to investigate the effect of *Malva sylvestris* leaf extract on rumen microbial populations, methane production and microbial fermentation of oat hay in sheep rumen liquor.

MATERIALS AND METHODS

Malva sylvestris plant was collected from Kermanshah province, Iran. For juice extraction, the fresh leaves of *Malva sylvestris* (pre-flowering) were finely grounded and blended in a commercial blender, squeezed through 2 layers of cheesecloth (Davys *et al.* 1969). The extract centrifuged at 454 × *g* for 15 minutes and the supernatant collected. The supernatant was saved and kept frozen at -80 ˚C until use.
The effects of four doses [0.0 (as control), 25, 50 and 100 µL] of *Malva sylvestris* leaf extract were examined *in vitro* using mixed ruminal microbiota from sheep rumen liquor. The substrate used for batch cultures was oaten hay (crude protein (CP)=15.5, neutral detergent fiber (NDF)=54.2 and acid detergent fiber (ADF)=29.4% of dry matter (DM)) wich was grounded to pass from 1 mm screen. Rumen content was obtained from three fistulated sheep (39±4.5 kg body weight) before morning feeding. Animals were fed 0.6 kg alfalfa hay and 0.6 kg concentrate (16.5% CP, 25% NDF and 45% nonfiber carbohydrates (NFC)). For eliminating of large feed particles, immediately rumen content was filtered through two layers of cheesecloth and transferred to the laboratory in a prewarmed thermos (38 ˚C). In the laboratory, under anaerobic conditions, 30 mL of buffered rumen fluid [ratio of rumen fluid to buffer was 1:2, buffer prepared as proposed by McDougall (1948)], using pipettor pump was added into 125 mL bottles containing 0.2 g of oaten hay (12 replicates for each treatment in two runs). Then, bottles were sealed by a rubber stopper and aluminum cap and placed in shaking water bath for 96 h at 38.6 ˚C. Accumulation of gas produced in the bottles, head space was measured by a pressure transducer at 2, 4, 8, 12, 18, 24, 36, 48, 72 and 96 h after the incubation and the gas released (Theodorou *et al.* 1995).

After 24 h of incubation 8 tubes from each treatment were withdrawn to determine *in vitro* truly degraded DM (INTDDM), partitioning factor (PF) and NDF degradability (NDFD) and enumeration of total viable and cellulolytic bacteria and rumen protozoa. Then each bottle content was filtered (42 µm pore size) and solid residues were used for determining IVDMD, PF and NDFD. A 10 mL sample of each filtrate bottle was taken and transfer into the separate flask (50 mL, 4 replicate/treatment). The flasks were closed and allowed to stand for 1 h at 38.6 ˚C. Rumen fluid was collected by suction from the middle of each flask. The ruminal fluid contained bacteria were serially diluted (10-fold increments) in the liquid version of mediums in the Hungate tubes (3 replicate). For enumeration of total viable and cellulolytic bacteria, the anaerobic technique proposed by Bryant (1972) was used for preparation medium. For cellulolytic bacteria ball-milled cellulose used as a single source of energy. The tubes were incubated at 38.6 ˚C for 72 h and 14 d (for cellulolytic bacteria) and in the end of incubation, growth was scored (+ or -) by the increase in optical density (650 nm). The cellulolytic and total viable bacteria population size was estimated using most probable number (MPN) procedure from replicate (3 tubes/dilution) dilutions. For protozoa enumeration, 5 mL of filtered rumen fluid was preserved using 5 mL of 50% Formalin (18.5% concentration of formaldehyde) as described by Dehority *et al*. (1984). Two drops of Brilliant green dye was added to 1 mL of rumen fluid (n=2) and stored overnight at the laboratory temperature. Then, 9 mL of 30% glycerol solution was added. Protozoa were enumerated microscopically in a Sedgwick-Rafter counting chamber. Protozoa species were identified from photographs and descriptions given by Ogimoto and Imai (1981).

Calculation and statistical analysis

Gas pressure was converted into volume using an experimentally calibrated curve. To estimate kinetic parameters of gas production, gas production data were fitted using France *et al*. (1993) equation as:

$$Y = A \times ((1\text{-exp})-(b(t\text{-}L))-(c(t^{½}\text{-}L^{½})))$$

Where:
A: volume of gas produced from quickly and slowly degradable fraction.
b and *c*: constants of the fractional rate (%/h).
t: incubation time (h).
L: lag time (h).
Y: volume of gas produced at time *t*.

The half-life ($t^{1/2}$, h) of the fermentable fraction of each substrate was calculated as the time taken for gas accumula-

tion to reach 50% of its asymptotic value. Methane production was measured according to the method proposed by Fievez *et al.* (2005).

True substrate degradability was measured following the procedure of Blummel *et al.* (1997). Briefly, the contents of the bottles were transferred into the Berzelius beaker by repeated washings with 50 mL of neutral detergent solution (double strength), refluxed for 1 h, filtered through silica crucibles (Grade 1) and then for determining organic matter (OM) concentration, residues were burnt in a furnace at 600 ˚C for 3 h.

Then INTDDM, INTDOM and NDFD were calculated as the ratio of residues DM or OM after 24 h of incubation/ incubated DM or OM. PF was calculated as the ratio of milligram organic matter truly degraded/mL gas produced after 24 h of incubation (Blummel *et al.* 1997).

The population size of total viable and cellulolytic bacteria were calculated using most-probable-number tables (Alexander, 1982) with values derived from the number of tubes that showed positive growth.

Data were analyzed using the SAS (SAS, 2002) and significance between individual means was identified using Duncan multiple range test. For enumeration total and cellulolytic bacteria and protozoa, least significant difference (LSD) was used to compare the means.

RESULTS AND DISCUSSION

In vitro rumen fermentation

Effect of addition of *Malva sylvestris* leaf extract on INTDDM, NDFD and PF of oat hay are summarized in Table 1. Relative to the control, the addition of *Malva sylvestris* leaf extract at 25, 50 and 100 µL/30 mL of medium resulted in a linear increase (P<0.01) in INTDDM and NDFD. Relative to the control, the PF was increased (P<0.05) with addition *Malva sylvestris* leaf extract at 25, 50 and 100 µL (2.74 *vs*. 2.81, 2.82 and 2.94).

As it is shown in Table 1, the addition of *Malva sylvestris* leaf extract at 25, 50 and 100 µL, after 24 h of incubation decreased (P<0.01) the methane production (except at 50 µL doses) relative to the control. Gas production not affected with *Malva sylvestris* leaf extract supplementation, except at 50 µL (41.22 *vs*. 40.16 in control, P-value=0.001). According to results shown in Table 2, the addition of *Malva sylvestris* leaf extract resulted in a decrease in half-life (*h*) of gas production at 25 and 50 µL and increase at 100 µL.

In addition, supplementation of *Malva sylvestris* at 25, 50 and 100 µL increased the lag time (*h*) and constant rate of gas production at half-life (*c*) (P<0.01). Half-life was decreased as a result of increased constant rate of gas production (*c*) during the half of incubation time (P<0.01).

Total viable and cellulolytic bacteria and protozoa population counting

Effect of *Malva sylvestris* leaf extract on genus diversity and total population of protozoa, total viable and cellulolytic bacteria population are shown in Tables 3 and 4. Results of the present study showed that the addition of *Malva sylvestris* leaf extract resulted in a decrease (P<0.01) in total protozoa (highest at 100 µL and lowest at 25 µL) and *Entodinium*, *Isotrichae*, *Diplodinium* and *Ophryoscolex genus*.

Relative to the control, the addition of *Malva sylvestris* leaf extract at 25 and 50 µL increased and at 100 µL decreased total viable and cellulolytic bacteria (P>0.05).

Partitioning factor (PF) is reported to be valuable in the accuracy of voluntary dry matter intake (DMI) prediction and microbial biomass synthesis efficiency of temperate-tropical crop residues and Mediterranean hays and forages with high PF result in high dry matter intake (DMI) (Blummel *et al.* 1997). The PF is known as the index of microbial biomass synthesis efficiency (Blummel *et al.* 1997). Therefore, results of the present study showed that *Malva sylvestris* leaf extract had a potential to increase the efficiency of ruminal microbial protein synthesis. The increase in PF by supplementation of *Malva sylvestris* leaf extract demonstrated that proportionally more of the truly degraded substrate was incorporated in microbial mass which can increase the efficiency of microbial protein synthesis. Blummel *et al.* (2005) reported 48.8% and 2.81 mg/mL for INTDDM and PF (at 24 h incubation) in oat hay, respectively.

The inhibitory effect of *Malva sylvestris* leaf extract on methane production confirmed their anti-methanogens potent on ruminal microbes involved in methanogenesis. In ruminants, methane emission represents 8 to 12% loss of intake energy (Johnson and Johnson, 1995). On the other hands, methane is a greenhouse gas that has a global warming potential 21 times that of CO_2 (Crutzen *et al.* 1995).

Therefore, supplementation of additives that decrease ruminal methane emission can increase the efficiency of energy utilization in ruminant and decrease environment contamination (Kim *et al.* 2013; Kim *et al.* 2015). In addition, utilization of low methane producing feeds that are available at livestock farms could be strategically considered to feed ruminants decreasing environmental impacts (Patra *et al.* 2015). Increase in gas production with the addition of 50 µL of *Malva sylvestris* leaf extract and decrease in gas production at 100 µL may be due to raise in the concentration of antimicrobial secondary compounds of *Malva sylvestris* that added to the medium at this level. Our findings agree with previous *in vitro* batch culture reports where high doses of essential oils or extract have been tested (Jahani-Azizabadi *et al.* 2011; Busquet *et al.* 2005).

Table 1 Effect of *Malva sylvestris* leaf extract on *in vitro* fermentation characteristics of oat hay

Items	*Malva sylvestris* leaf extract (µL)				SEM	P-value
	0.0	25	50	100		
Gas production (mL/24 h/0.2 g)	40.16^{b}	40.16^{b}	41.22^{a}	40.03^{b}	0.15	0.001
Methane production (mL)	11.82^{b}	11.02^{c}	12.32^{a}	10.75^{d}	0.19	0.001
Methane reduction potential (%)	-	6.34^{b}	-12.29^{c}	13.10^{a}	2.82	0.001
IVTDMD (g/kg DM)	527.10^{d}	534.60^{c}	542.20^{b}	573.30^{a}	5.33	0.001
NDFD (g/kg DM)	615.30^{b}	623.20^{b}	625.30^{b}	674.30^{a}	8.66	0.03
PF (mg/mL)	2.74^{c}	2.81^{b}	2.82^{b}	2.92^{a}	0.02	0.001

IVTDMD: *in vitro* truly degraded dry matter; NDFD: nuteral detergent fiber degradability; PF: partitioning factor and DM: dry matter.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 2 Effect of *Malva sylvestris* leaf extract on *in vitro* gas production parameters

Parameters	*Malva sylvestris* leaf extract (µL)				SEM	P-value
	0.0	25	50	100		
A (mL/0.2 g DM)	60.57^{b}	60.04^{c}	56.43^{d}	61.12^{a}	0.05	0.001
b (mL/h)	0.06^{a}	0.05^{b}	0.05^{b}	0.06^{a}	0.002	0.001
c (mL/h$^{1/2}$)	0.03^{b}	0.05^{a}	0.04^{a}	0.04^{a}	0.004	0.002
L (h)	0.61^{c}	0.95^{b}	1.01^{b}	1.13^{a}	0.06	0.002
Half-life (h)	11.13^{b}	11.05^{c}	10.50	11.30^{a}	0.01	0.001
Fermentation rate (T$^{1/2}$/h)	0.011^{b}	0.013^{b}	0.016^{a}	0.007^{c}	0.001	0.001

A: potential gas production; b,c: constant rate; L: lag time and DM: dry matter.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 3 Effect of *Malva sylvestris* leaf extract supplementation on *in vitro* protozoa population (×10^{5}/mL)

Protozoa population	*Malva sylvestris* leaf extract (µL)				SEM	P-value
	0.0	25	50	100		
Total	2.89^{a}	2.62^{b}	2.14^{c}	1.17^{d}	0.20	0.001
Entodinium	2.34^{a}	2.23^{a}	1.88^{b}	0.94^{c}	0.17	0.001
Isotrichae	0.20^{a}	0.10^{b}	0.05^{c}	0.12^{b}	0.02	0.001
Diplodinium	0.05^{a}	0.05^{a}	0.03^{b}	ND	0.006	0.001
Ophryoscolex	0.17^{a}	0.11^{b}	0.05^{c}	0.03^{d}	0.02	0.001

ND: not detected.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 4 Effect of *Malva sylvestris* leaf extract on *in vitro* total and cellulolytic bacteria (log10/mL of medium)

Items	*Malva sylvestris* leaf extract (µL)				SEM	P-value
	0.0	25	50	100		
Total viable bacteria	8.1	8.56	8.58	7.70	0.35	0.08
Cellulolytic	7.1	7.21	7.49	6.95	0.15	NS

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
NS: non significant.
SEM: standard error of the means.

Linearly increasing in lag time withe increase in the concentration of *Malva sylvestris* leaf extract could be due to the increase in time required for bacterial colonization on feed particles. Therefore, results of the present study demonstrated that high doses of *Malva sylvestris* leaf extract in high-forage diets can decrease in dry matter intake due to increase in colonization time and rumen filling.

Results of the present study demonstrated that *Entodinium* species accounted for around 90% of total counted protozoa. This finding was in agreement with values observed by other researchers (Santra *et al*. 1998; Hindrichsen *et al*. 2002).

In addition, results of the present study suggested that *Entodinium*, *Diplodinum* and *Ophryoscolex* species were more sensitive to the addition of *Malva sylvestris* leaf extract. Relative to the control, abundance of *Entodinium*, *Diplodinum* and *Ophryoscolex* species were decreased by 59.8%, near to 100% and 82.3%, respectively. Therefore, in the present study decrease in methane production with addition of *Malva sylvestris* leaf extract was accompanied by a significant decrease in protozoa count. Around 10-20% of ruminal methanogenic bacteria are attached to the surface of protozoa, especially *Entodinium* species (Stumm *et al*. 1982).

The methanogens take up hydrogen, which appears too toxic for *Entodinium* species activity. Previous studies have demonstrated that rumen protozoa defaunation reduced methane production ranging from 13 to 35% (Morgavi *et al.* 2008; Morgavi *et al.* 2012) and 9 to 25% *in vitro* (Newbold *et al.* 1995).

Neutral detergent fiber degradability (NDFD) is an important index of forage quality. Increased NDFD may result to greater voluntary feed intake by reducing physical fill in the rumen (Dado and Allen, 1995). *In vitro* or *in situ* one unit increase in forage NDFD was associated with 0.17 kg increase in dry matter intake (Oba and Allen, 1999).On the other hand, results of the present study showed that addition of *Malva sylvestris* leaf extract resulted in a significant linear ($P<0.01$) increase in NDF disappearance. No significant changes in rumen cellulolytic bacteria and increase in NDF disappearance of oaten hay suggest that probably, the addition of *Malva sylvestris* leaf extract could result in an increase in the relative abundance of obligate cellulolytic bacteria with higher cellulolytic activity and persistence to main secondary compounds of *Malva sylvestris* leaf extract. However, unfortunately, there is not any information about the effect of *Malva sylvestris* leaf extract on rumen microbial fermentation.

CONCLUSION

In conclusions, *Malva sylvestris* leaf extract used in the present study resulted in the valuable effect on rumen microbial fermentation. For determine the mechanisms that *Malva sylvestris* leaf extract affects ruminal degradation of NDF and methane emission, more studies on the main rumen cellulolytic and methanogens bacteria are needed. As regards that this is first report about effect of *Malva sylvestris* extract on rumen microbial fermentation, future research is required to determine optimal doses and the effect of *Malva sylvestris* organic extract and essential oil on *in vitro* and *in vivo* rumen microbial fermentation patterns and animal performance.

ACKNOWLEDGEMENT

The authors thank the University of Razi (Kermanshah, Iran) for the financial support.

REFERENCES

Alexander M. (1982). Most probable number method for microbial populations. Pp. 815-820 in Methods of Soil Analysis, Part 2. A.L. Page, R.H. Miller and D.R. Keeney, Eds. American Society of Agronomy, Madison, USA.

Blummel M., Cone J.W., Van Gelder A.H., Nshalai I., Umunna N.N., Makkar H.P.S. and Becker K. (2005). Prediction of forage intake using *in vitro* gas production methods comparison of multiphase fermentation kinetics measured in an automated gas test and combined gas volume and substrate degradability measurements in a manual syringe system. *Anim. Feed Sci. Technol.* **5,** 123-124.

Blummel M., Makkar H.P.S. and Becker K. (1997). *In vitro* gas production: a technique revisited. *J. Anim. Physiol. Anim. Nutri.* **77,** 24-34.

Bryant M.P. (1972). Commentary on the Hungate technique for culture of anaerobic bacteria. *Am. J. Clin. Nutr.* **25(12),** 1324-1328.

Busquet M., Calsamiglia S., Ferret A. and Kamel C. (2005). Screening for effects of plant extracts and active compounds of plants on dairy cattle rumen microbial fermentation in a continuous culture system. *Anim. Feed Sci. Technol.* **124,** 597-613.

Crutzen P.J. (1995). The role of methane in atmospheric chemistry and climate. Pp. 291-315 in Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction. W.V. Engelhardt, S. Leonhard-Marek, G. Breves and D. Giesecke, Eds. Ferdinand Enke Verlag Stuttgart, Germany.

Dado R.G. and Allen M.S. (1995). Intake limitation, feeding behaviour and rumen function of cows challenged with rumen fill from dietary fiber or inert bulk. *J. Dairy Sci.* **78,** 118-113.

Davys M.N.G., Pirie N.W. and Street G. (1969). A laboratory-scale press for extracting juice from leaf pulp. *Biotech. Bioeng.* **11,** 529-538.

Dehority B.A. (1984). Evaluation of subsampling and fixation procedures used for counting rumen protozoa. *Appl. Environ. Microbiol.* **48,** 182-185.

Durmic Z., Moate P.L., Eckard R., Revell D.K., Williams R. and Vercoe P. (2014). *In vitro* screening of selected feed additives, plant essential oils and plant extracts for rumen methane mitigation. *J. Sci. Food Agric.* **94,** 1191-1196.

European Union. (2003). Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on Additives for Use in Animal Nutrition. Council of the European Union , European Parliament.

Fievez V., Babayemi O.J. and Demeyer D. (2005). Estimation of direct and indirect gas production in syringes: a tool to estimate short chain fatty acid production that requires minimal laboratory facilities. *Anim. Feed Sci. Technol.* **123,** 197-210.

France J., Dhanoa M.S., Theodorou M.K., Lister S.J., Davies D.R. and Isaac D.A. (1993). Model to interpret gas accumulation profiles associated with *in vitro* degradation of ruminant feeds. *J. Theor. Biol.* **163,** 99-111.

Gasparetto J.C., Martins A.F., Hayashi S.S., Otuky M.F. and Pontarolo R. (2011). Ethnobotanical and scientific aspects of *Malva sylvestris*: a millennial herbal medicine. *J. Pharm. Pharmacol.* **64**, 172-182.

Gonzalez J.A., Barriuso M.G. and Amich F. (2010). Ethnobotanical study of medicinal plants traditionally used in the Arribesdel Duero, western Spain. *J. Ethnopharmacol*. **131,** 343-355.

Hindrichsen I.K., Osuji P.O., Odenyo A.A., Madsen J. and Hvelplund T. (2002). Effects of supplementation of a basal diet of maize stover with different amounts of

*Leucaenadiversifolia*on intake, digestibility and nitrogen metabolism and rumen parameters in sheep. *Anim. Feed Sci. Technol*. **98,** 131-142.

Iason G. (2005). The role of plant secondary metabolites in mammalian herbivory: ecological perspectives. *Proc. Nutr. Soci.* **64,** 123-131.

Idolo M., Motti R. and Mazzoleni S. (2010). Ethnobotanical and phytomedicinal knowledge in a long history protected area, the Abruzzo, Lazio and Molise National Park (Italian Apennines). *J. Ethnopharmacol.* **127,** 379-395.

Jahani-Azizabadi H., Mesgaran M.D.,Vakili A.R., Rezayazdi K. and Hashemi M. (2011). Effect of various medicinal plant essential oils obtained from semi-aridclimate on rumen fermentation characteristics of a high forage diet using *in vitro* batch culture. *African J. Microbiol. Res.* **5,** 4812-4819.

Johnson K.A. and Johnson D.E. (1995). Methane emission from cattle. *J. Anim. Sci.* **73,** 2483-2492.

Kim E.T., Guan Le L., Lee S.J., Lee S.M., Lee S.S., Lee I.D., Lee S.K. and Lee S.S. (2015). Effects of flavonoid-rich plant extracts on *in vitro* ruminal methanogenesis, microbial populations and fermentation characteristics. *Asian-Australas J. Anim. Sci.* **28,** 530-537.

Kim E.T., Min K.S., Kim C.H., Moon Y.H., Kim S.C. and Lee S.S. (2013). The effect of plant extracts on *in vitro* ruminal fermentation, methanogenesis and methane-related microbes in the rumen. *Asian-Australas J. Anim. Sci.* **26,** 517-522.

McDougall E.I. (1948). Studies on ruminant saliva. 1. The composition and output of sheeps saliva. *Biochem. J*. **43,** 99-109.

Morgavi D.P., Forano E., Martin C. and Newbold C.J. (2008). Microbial ecosystem and methanogenesis in ruminants. *Animal*. **4,** 1024-1036.

Morgavi D.P., Martin C., Jouany J.P. and Ranilla M.J. (2012). Rumen protozoa and methanogenesis: not a simple cause-effect relationship. *Br. J. Nutr.* **107,** 388-397.

Newbold C.J., Lassalas B. and Jouany J.P. (1995). The importance of methanogens associated with ciliate protozoa in ruminal methane production *in vitro*. *Lett. Appl. Microbiol.* **21,** 230-234.

Oba M. and Allen M.S. (1999). Evaluation of the importance of NDF digestibility: effects on dry matter intake and milk yield of dairy cows. *J. Dairy Sci.* **82,** 589-596.

Ogimoto K. and Imai S. (1981). Atlas of Rumen Microbiology. Japanese Science Society Press, Tokyo, Japan.

Patra P.K. and Sahoo A.K. (2015). Evaluation of feeds from tropical origin for *in vitro* methane production potential and rumen fermentation *in vitro*. *Spanian J. Agric. Res.* **13,** 1-12.

Santra A., Karim S.A., Mishra A.S., Chaturvedi O.H. and Prasad R. (1998). Rumenciliate protozoa and fibre digestion in sheep and goats. *Smal Rumin. Res.* **30,** 13-18.

SAS. (2002). SAS®/STAT Software, Release 9. SAS Institute, Inc., Cary, NC. USA.

Stumm C.K., Gijzen H.J. and Vogels G.D. (1982). Association of methanogenic bacteria with ovine rumen ciliates. *Br. J. Nutr.* **47,** 95-99.

Theodorou M.K., Williams B.A., Dhanoa M.S., McAllan A.B. and France J. (1995). A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Anim. Feed Sci. Technol.* **48,** 185-197.

Wallace R.J. (2004). Anti-microbial properties of plant secondary metabolites. *Proc. Nutr. Soc.* **63,** 621-629.