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RESEARCH ARTICLE

Effects of Purified Saponin on Rumen Methanogenesis and Rumen Fermentation Characteristics Studied Using *In Vitro* Gas Production Technique

[1]A. Bharathidhasan, [2]K. Viswanathan, [3]V. Balakrishnan, [4]C. Valli, [5]S. Ramesh and [6]S. M. A. Senthilkumar

[1,3,4]Department of Animal Nutrition, [5]Department of Veterinary Pharmacology and Toxicology, [6]Department of Animal Biotechnology, Madras Veterinary College, Chennai-600 007, [2]Institute of Animal Nutrition, Kattupakkam-603 203, Tamil Nadu Veterinary and Animal Sciences University, Chennai-600 051, Tamil Nadu, India

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***Corresponding Author**
A Bharathidhasan
bharathidhasana@tanuvas.org.in

ABSTRACT

An experiment was conducted to study the effects of purified saponin at the level of 0, 1.55, 3.10, 4.65 and 6.20 mg/30 ml rumen inoculum in triplicate on rumen methanogenesis and rumen fermentation characteristics by *in vitro* gas production technique. The total gas production was significantly (P<0.01) reduced in all saponin treated groups when compared to the control. The highest level of reduction in total gas was observed with 4.65 mg saponin treatment than other treatment groups. The methane production was significantly (P<0.01) decreased in all saponin treated groups when compared to the control. The highest level of methane reduction was observed with 6.2 mg saponin treatment than other treatment groups. The decreases in methane reduction were 14.04 %, 21.90 %, 34.30 % and 37.60 % in 1.55, 3.10, 4.65 and 6.20 mg saponin levels respectively than 0 mg saponin level. The percentage of methane on total gas production was also significantly (P<0.01) reduced in all saponin treated groups than the control. The highly significant (P<0.01) reduction was observed with 4.65 mg of saponin treatment and this treatment decreased the methane percentage on total gas production by 15.04 % when compared to the other treatment groups. The methane emission per 100 mg of digested substrate was also significantly (P<0.01) reduced with 4.65 mg saponin treatment than other treatment groups. No significant difference was observed on pH and ammonia nitrogen levels in all the treatment groups. The *in vitro* true dry matter digestibility (*IVTDMD*) was not affected by saponin and no significant difference was observed. The protozoal count was significantly (P<0.05) reduced in all saponin treated groups when compared to the control. The reduction in protozoal counts were 14.40%, 15.56%, 19.31% and 23.05% respectively with 1.55, 3.10, 4.65 and 6.20 mg saponin treatments when compared to the control. The bacterial count in all saponin treated groups was not significantly affected, however the bacterial counts showed a decreasing trend with increased levels of saponin when compared to the control. No significant difference was observed on total volatile fatty acids (TVFA), acetic acid, propionic acid, butyric acid and acetate to propionate ratio in all saponin treated groups when compared to the control. Based on our findings we concluded that treatment with purified saponin reduces the total gas and methane emission significantly without affecting the rumen fermentation characteristics such as pH, ammonia nitrogen, *IVTDMD*, microbial load and TVFA.

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INTRODUCTION

Methane is the most important gas among the greenhouse gases that are responsible for global warming (IPCC, 2001). Ruminants produce methane under anaerobic fermentation as a path way for the disposal of metabolic hydrogen ion produced during microbial fermentation. The estimated methane emission from enteric fermentation was 15–20 % of the global methane production (Moss *et al*., 2000) and it increases the global temperature. Increased temperature due to global warming has an adverse impact on global weather patterns. Another adverse impact of methane production is that it leads to a loss of feed energy by 8-12 % and thus lowers the efficiency of animal production. Therefore decreasing methane production is desirable not only for reducing greenhouse gases and global warming but also to improve the efficiency of animal production. Recently, researchers have emphasized on the reduction of methane emission by using various feeding strategies (Singh and Madhu Mohini, 2004; Bakshi *et al*., 2004; Iqbal *et al*., 2008; Patra and Saxena, 2010; Bodas *et al*., 2012). Modification of the diet has been employed to mitigate methane emissions. For example antibiotics like ionophore compounds such as monensin, lasolocid and many other chemical feed additives have been shown to decrease methane production in ruminants (Agarwal *et al*., 2006). However, these chemical feed additives are either toxic to host animals (Moss *et al*., 2000) or an increasing awareness of hazards associated with the food residues derived from animal tissues has been diverted to utilize plant secondary metabolites. Also development of resistance while using antibiotics in ruminants will compels the utilization of natural plant source like saponin for reduction of methane emission.

Saponin is one of the important plant secondary metabolite which has been shown to selectively modulate the rumen microbial population resulting in an improvement of rumen fermentation and to decrease the methane production (Patra and Saxena, 2010; Bodes *et al*., 2012). The majority of research on saponin has been focused on exploiting its characteristics that inhibit rumen ciliate protozoa and on its ability to selectively inhibit some bacteria and thus reduces the production of hydrogen ions needed for the methane emission (Patra and Saxena, 2009). A number of studies have reported that plants rich in saponins decreased the methane production in the rumen (Hu *et al*., 2005; Patra and Saxena, 2010; Bodas *et al*., 2012). Similarly Goel *et al*. (2008) who observed that the methane emission was decreased via reducing the methanogen populations, using *Sesbania saponins* by 78 %, *Fenugreek saponins* by 22 % and *Knautia saponins* by 21% in the *in vitro* fermentation technique. Saponin extracts from *Yucca schidigera* and *Quillaja saponaria* had been demonstrated to reduce methanogenesis *in vitro* (Pen *et al*., 2008; Holtshausen *et al*., 2009). Wina *et al*. (2005) also reported that the methanol extract of *Sesbania saponaria* reduced the numbers of protozoa completely at the concentration of 1 mg/ml. Sterol binding capability of saponins has been implicated in the destruction of protozoal cell membranes (Hostettmann and Marston, 1995). Thus saponin appears to reduce methane emission by inhibiting protozoa, which reduces the availability of hydrogen ions for methane production. Hence the present study was carried out to identify the effects of purified saponin on rumen methane production and rumen fermentation characteristics by *in vitro* gas production technique. Our findings reveal that purified saponin significantly reduces the total gas and methane emission without affecting the rumen fermentation characteristics.

MATERIALS AND METHODS

The *in vitro* gas production study was carried out according to the protocol described earlier (Menke and Steingass, 1988). Our study was aimed at evaluating the effects of purified saponin at different levels viz. 1.55, 3.10, 4.65 and 6.20 mg/30ml rumen inoculums in triplicate (0, 0.775%, 1.55%, 2.325% and 3.1% of substrate) on rumen methanogenesis and rumen fermentation characteristics. The purified saponin purchased from M/s. Sigma Aldrich (CAS No.8047-15-2; EC No.232-462-6; MDL No.MFCD00081981) and the substrate Hybrid Cumbu Nappier grasses (*Pennisetum typhoides x Pennisetum purpureumx*) were used for this study.

In vitro gas production study

The *in vitro* gas production study was carried out with rumen fluid collected from three cattle maintained on grazing and it was squeezed through four layers of gauze in to an Erlenmeyer flask under continuous flushing with CO_2 and it was maintained at the temperature of 39 ^oC. Then rumen fluid was mixed with media as described by Menke and Steingass (1988). The substrate Hybrid Cumbu Nappier grass (CO4 grass variety) was dried and milled to pass through 1 mm sieve and 200 mg was weighed and taken in 100 ml calibrated syringes and weighed quantity of saponin at 0, 1.55, 3.10, 4.65 and 6.20 mg were added to the syringes in triplicate. Then 30 ml of rumen inoculum was anaerobically transferred to glass syringe and it was incubated in a shaking water bath at 39 ^oC for 24 hrs.

At the end of the incubation period, the total gas was measured along with the pH in the fermentation fluid. The gas samples were collected in vacuotainer for estimation of methane while the fermented fluid was collected for the estimation of ammonia nitrogen, volatile fatty acids, true dry matter digestibility, bacterial and protozoal count.

Estimation of methane

Methane concentration was estimated using Gas Chromatography (Perkin Elmer, Claurus 500 model) fitted with Flame Ionization Detector (FID) and capillary column (30 meter length and 250 micrometer diameter). Helium was used as a carrier gas with oven temperature at 60^o C, injector temperature at 100^{o}C and detector temperature at 110^{o}C. Methane concentration in the samples (%) was calculated using the following formula.

Methane concentration (%) = Peak area of sample gas/ Peak area of standard gas x Methane concentration in standard (%)

Methane emission (ml) = Methane concentration (%)/100 x Net gas production (ml)

Estimation of volatile fatty acids

The volatile fatty acids were estimated as per the method of Chase (1990). 2.5ml of fermented media from each syringe was mixed with 0.5ml of 25% meta phosphoric acid and centrifuged at 20000g for 30 minutes at 4°C and the clear supernatant was collected in gas chromatography vials. The 1µl of supernatant was injected into Gas Chromatography (Perkin Elmer, Claurus 500 model) fitted with Flame Ionization Detector (FID) and capillary column (30 meter length and 250 micrometer diameter). Helium was used as a carrier gas with oven temperature at 175° C, injector temperature at 220°C and detector temperature at 240°C.

Estimation of *in vitro* true dry matter digestibility (*IVTDMD*)

After incubation the fermented fluid was centrifuged and the residue or pellet was transferred into the glass crucible and fitted in Fibretec and 100 ml of Neutral Detergent Solution (NDS) was added. Then it was refluxed for one hour and the residue was recovered. The true digestibility was calculated as the weight of substrate incubated minus the weight of the residue after NDS treatment (Van Soest and Robertson, 1988). The methane emission per 100 mg of true digestible substrate was also estimated.

Total Bacterial count

Total bacterial count was carried out using gram's staining as per the method of Gall e*t al*, (1949). One ml of effluent fluid was diluted to 5 ml with 10% formal saline. One ml of diluted fluid was again diluted with 10% formal saline to 100 ml. 0.01ml of this fluid spread over marked area of 1 SQCM on a clean glass slide. The smear was air and flame dried and stained with gram's staining. The bacteria were counted in 30 fields covering all the sections of the smear. The total bacterial load/ml effluent is calculated as shown below,

Area of each field: πr^2 'r' – Radius of the microscopic fluid measured by using micrometer
Area of the smear: 1 SQCM (or) 100 SQMM
Number of fields/100 SQMM: 100/ πr^2 = H
Total number of fields counted: 30
Average number of bacteria/field: N
Volume of effluent fluid used: 0.00002 ml

No. of bacteria in 1 ml of effluent fluid = H x N x 10^5 / 2

Total Protozoal count

Total protozoal count was calculated using the technique described previously (Moir, 1951). 1 ml of effluent fluid was diluted to 5 ml with 10 % formal saline. 2 % Eosin stain was added to the diluted fluid at the rate of one drop per 5 ml. After allowing for 5-10 minutes for the protozoa to take up the stain, the contents were mixed thoroughly and the haemocytometer was charged. The protozoa were counted in the entire eight WBC chamber. The total protozoal count/ml effluent was calculated as shown below,
Dilution ratio: 1:5
Volume of 1 WBC chamber: 0.0001 cmm
0.0001 cmm of diluted effluent contained: A protozoa
No. of protozoa in 1 ml of effluent fluid = A x 5 / 0.0001 = A x 5000

Estimation of ammonia nitrogen

The ammonia nitrogen was estimated by steam distillation as per the method of Makkar and Becker (1996). The data collected on various parameters was statistically analyzed using one way anova as per the method of Snedecor and Cochran (1989).

RESULTS

The effects of saponin on total gas, methane, *IVTDMD*, total nitrogen, total volatile fatty acids, acetic acid, propionic acid, butyric acid and acetate to propionate ratio by *in vitro* gas production technique were presented in Table 1.

Effects of purified saponin on total gas and methane emission

The total gas production was significantly (P<0.01) reduced in all the treatment groups when compared to the control. The highest level of reduction was observed with 4.65 mg saponin level than control.

The methane production was significantly (P<0.01) decreased in all saponin treatment groups than control. The highest level of methane reduction was observed with 6.2 mg saponin level than other treatment groups. The decrease in methane reductions were 14.04 %, 21.90 %, 34.30 % and 37.60 % in 1.55, 3.10, 4.65 and 6.20 mg saponin levels, respectively when compared to the control. The percentage of methane on total gas production was significantly (P<0.01) decreased in all saponin treated groups than control. Methane reduction on total gas was highest with 4.65 mg of saponin level (P<0.01) and this treatment decreased the methane production by 15.04 % when compared to 0 mg saponin level. The methane emission per 100 mg of truly digested substrate was significantly (P<0.01) reduced with 4.65 mg saponin level than control.

Furthermore, the CO_2 level was also significantly (P<0.01) reduced in all treatment groups than control. The highest reduction was observed with 3.1 mg of saponin level when compared to other treatment groups.

Effects of purified saponin on pH, ammonia nitrogen and *IVTDMD*

No significant difference was observed on the pH in all treatment groups. However the pH levels showed a decreasing trend in all saponin treated groups than control. Similarly the ammonia nitrogen content in all saponin treated groups was not significantly different and it was marginally reduced in all saponin treated groups than control. The *IVTDMD* was not affected by saponin and no significant difference was observed (P<0.05).

Effects of purified saponin on bacterial and protozoal counts

The bacterial count in all saponin treated groups was not significantly different when compared to the control. Bacterial load was reduced marginally in all saponin treated groups when compared to the control. The protozoal count was significantly (P<0.05) reduced in all

Table 1: Effects of purified saponin at varying levels on rumen methanogenesis and rumen fermentation characteristics (Values are reported as Mean ± S.E)

Parameters	Purified saponin level (mg/30 ml rumen inoculum)				
	0 mg(0% of substrate)	1.55 mg (0.775% of substrate)	3.1 mg (1.55% of substrate)	4.65 mg (2.325% of substrate)	6.2 mg (3.1% of substrate)
Total gas (ml)**	12.27±0.19^{d}	10.60±0.06^{c}	9.91±0.21 ^{b}	9.47±0.20^{a}	8.97±0.12 ^{a}
Methane (ml)**	2.42±0.01^{d}	2.08±0.03^{c}	1.89±0.04 ^{b}	1.59±0.03^{b}	1.51±0.01 ^{a}
CO2 (ml)**	9.84±0.19^{c}	8.52±0.09^{b}	8.01±0.17 ^{ab}	7.88±0.18^{a}	7.46±0.12 ^{a}
Percentage of methane emission on Total gas production**	19.75±0.37^{b}	19.66±0.43^{b}	19.11±0.22 ^{b}	16.78±0.13^{a}	16.81±0.24 ^{a}
Methane(ml)/100 mg of truly digested substrate **	2.02±0.02^{d}	1.76±0.04^{c}	1.60±0.03 ^{b}	1.35±0.03^{a}	1.26± 0.01 ^{a}
pH ^{NS}	7.05±0.08	6.94±0.14	7.04±0.12	6.93±0.09	7.00±0.15
IVTDMD (%)^{NS}	59.93±0.43	59.27±0.46	59.20±0.95	59.03±0.44	59.63±0.24
Ammonia Nitrogen (mg/100ml) ^{NS}	38.22±0.49	37.33±0.93	36.40±1.62	35.47±1.23	35.37±1.50
Bacterial count (X 10^{8}) ^{NS}	4.13±0.12	3.97±0.07	3.93±0.09	3.90±0.06	3.80±0.27
Protozoal count (X 10^{5})*	3.47±0.03^{b}	2.97±0.12^{a}	2.93±0.26 ^{a}	2.80±0.06^{a}	2.67±0.12 ^{a}
TVFA (mg/dl) ^{NS}	65.79±2.02	65.69±2.32	65.82±2.15	64.91±2.32	67.41±3.31
Acetic acid (%)^{NS}	73.43±0.46	72.94±1.27	72.84±0.91	73.27±1.21	73.50±0.81
Propionic acid (%)^{NS}	19.58±0.52	19.50±1.10	19.25±1.06	19.42±1.30	19.38±1.43
Butyric acid (%)^{NS}	6.99±0.16	7.56±0.37	7.91±0.48	7.31±0.70	7.12±0.84
A/P ratio ^{NS}	3.76±0.12	3.77±0.29	3.81±0.26	3.82±0.33	3.84±0.34

NS – Non significant; Mean of three observations; Means with at least one common superscript (a, b, c, d) in a row do not differ significantly (P<0.05)*, (P<0.01) **

saponin treated groups when compared to the control. The reduction in protozoal count was 14.4 %, 15.56 %, 19.31 % and 23.05 % in 1.55, 3.10, 4.65 and 6.20 mg/30 ml of saponin levels, respectively than control.

Effects of purified saponin on total volatile fatty acids (TVFA), acetic acid, propionic acid, butyric acid and acetate to propionate ratio

There was no significant difference observed by the addition of saponin on total volatile fatty acids (TVFA), acetic acid, propionic acid, butyric acid and acetate to propionate ratio in all treatment groups.

DISCUSSION

As discussed below, our study provides the evidence to show that saponin has a beneficial effect of reducing the total gas and methane levels without any deleterious effects on the rumen fermentation characteristics.

Effects of purified saponin on total gas and methane emission

As observed in the present study, Makkar *et al*. (1998a) also observed that saponin from *Acacia auriculoformis* decreased the total gas production. However the saponin from *Quillaja saponaria* did not affect the gas production (Makkar *et al*. (1998a). There is evidence to show that, the total gas production was decreased by the supplementation of saponin based surfactants (Wang *et al*., 2011). Feng *et al*. (2012) also reported that total gas levels decreased numerically with increasing levels (0.3, 0.6 and 0.9 g/litter) of saponin when compared to the control.

Our data shows that the methane emission decreased by 14.04 % to 37.60 % as the level of saponin increased from 1.55 mg to 6.2 mg. Similarly, Lila *et al*. (2003) reported that the methane emission decreased by 18 - 52% as the level of saponin increased from 1.2 mg to 3.2 mg/L. Saponin from plants such as *Yucca shidigera*, *Quillajia saponaria* and *Acacia concinna* (Patra *et al*., 2006b) *Sapondis mukorassi* fruit pulps (Agarwal *et al*., 2006), *Knautia arvensis* leaves and *Sesbania sesban* leaves (Goel *et al*., 2008b) have been shown to decrease methane production. Further Guo *et al*., (2008) observed that the saponin level at 0.4mg/ml significantly (P<0.01) reduced methane production by 76 % than control by *in vitro* study. Holtshausen *et al*., (2009) was also observed that *Yucca schidigera* plant extract containing 6 % saponin added at the level of 0.38 g /liter reduces the methane emission by 8.5 % than control. Feng *et al*., (2012) also observed that addition with 0.3, 0.6, 0.9 g/litter of saponin significantly (P<0.05) reduced methane concentration by 23.43, 24.93 and 25.30 % respectively by *in vitro* gas production technique.

Our observation that the methane emission per 100 mg of truly digested substrate was reduced in 4.65 mg saponin level agrees well with Castro-Montyo *et al*. (2011) who also reported that the addition of *Quillaja Saponin* reduced the methane by 4.1 ml/100 mg substrate at 1.25 mg of saponin/ litter by *in vitro* studies. Feng *et al*. (2012) also observed that the methane concentration in mM per 200 mg of dry matter decreased significantly (P<0.05) by 26.67, 28.89, 31.11% respectively in 0.3, 0.6, 0.9 g/litter of saponin level.

It has been suggested that saponin may decrease the protozoal numbers which leads to reduce the availability of hydrogen ions for methane production by methanogen (Patra and Saxena, 2009). Furthermore it has been show that the saponins reduced methane production via diminished activity of methane producing gene without changing the total methanogen population (Guo *et al*., 2008).

Effects of purified saponin on pH, ammonia nitrogen and *IVTDMD*

Our result is in agreement with the earlier study by Hanim *et al*. (2009) who observed that saponin had no effect on pH. This result is further supported by the study of Feng *et al*. (2012) who also reported that the addition of saponin did not influence the pH of medium

Though it was not significant, our finding that the ammonia nitrogen content was marginally reduced in all

saponin treated groups than control. It was consistent with earlier reports by Williams and Coleman (1991) reported that ammonia nitrogen concentration in the rumen was unchanged when protozoal counts were inhibited. In contrary to the present findings by Feng *et al*. (2012), observed that the two levels of saponin (0.6, 0.9 g/L) were significantly (P<0.05) reduced the ammonia nitrogen concentration as compared to control.

On contrary to the present study on *IVTDMD*, Wang *et al*. (2011) reported that the supplementation of surfactant saponin at the levels of 5-20 µl/g dry matter linearly increased apparent dry matter digestibility at 12 and 24 hrs interval.

Effects of purified saponin on bacterial and protozoal counts

In the present study the bacterial load was reduced marginally in all the saponin treated groups. This findings agrees well with Wallace *et al*. (1994) who reported that saponins inhibit the growth of *Butyrivibrio fibrisolvens* and *Strptococcus bovis* bacteria. It seems that saponins show a more marked antibacterial activity against Gram positive than against Gram negative bacteria (Patra and Saxaena, 2009).

The effect of saponins to decrease the protozoal count as observed in this study agrees with earlier study by Istiqomah *et al*., (2011) who showed that the protozoal number significantly decreased by 43.08 % at 10 % saponin level than control. Increasing concentration of gross saponin of *Tribulus terrestris* (0.15, 0.3, 0.6, 0.9 g/L) resulted in a significant decrease in total protozoal counts (Feng *et al*., 2012). Also saponin and saponin containing extracts have significant inhibitory effects on protozoa (Pen *et al*., 2008 and Guo *et al*., 2008). The mechanism of protozoal lysis is suggested by the change in the cell membrane permeability as they form complexes with cholesterol in the protozoal cell membrane and result in cell lysis which in turn decreases the hydrogen ion transfer and ultimately reduces the methane production (Francis et al., 2002).

Effects of purified saponin on total volatile fatty acids (TVFA), acetic acid, propionic acid, butyric acid and acetate to propionate ratio

As observed in the present study Feng *et al*., (2012) also reported that the saponin addition did not influence the TVFA concentrations significantly. Other studies have shown that *Quillaja saponin* (Makkar and Becker, 1996), Yucca extract (Wang *et al*,1998) and tea saponin (Hu *et al*.,2005) did not influence the level of TVFA. In contrary to the present findings, Lila *et al*, (2003) observed an increase in TVFA concentrations by the addition of sarsaponin in *in vitro* gas production technique. Istiqomah *et al*. (2011) also reported that the VFA concentration increased at 10% saponin level than other treatment groups. On contrary to the present study on unaltered acetate to propionate ratio, acetic acid and propionic acid level in the fermented medium, Istiqomah *et al*. (2011) found that ratio of acetate to propionate ratio decreased at 5, 10 and 15 % saponin level. Similarly Feng *et al*. (2012) also observed that the saponin level at 0.9g/L decreased the acetic acid and at 0.6 and 0.9 g/L increased the propionic acid concentration significantly when compared to the control.

Conclusion

It was concluded that the purified saponin significantly (P<0.01) reduced the methane production by 37.6% at 6.2 mg/30 ml rumen inoculum than control. There was no significant difference was observed in pH, *IVTDMD*, ammonia nitrogen and volatile fatty acids. However the protozoal number significantly (P<0.05) decreased indicating that cell lysis of protozoa led to a reduced production of hydrogen ion required for methane production. Our study provides explicit evidence to show that the use of saponins has a beneficial effect as it significantly depresses the methane production without having a negative effect on the rumen fermentation process.

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