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Effect of Gross Saponin of *Tribulus terrestris* on Ruminal Fermentation and Methane Production *in vitro*

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Abstract: The objective of this study was to ascertain the effect of various levels of Gross Saponin of *Tribulus terrestris* (GSTT) supplementation at 0, 0.15, 0.30, 0.60 and 0.90 g L^{-1} of incubation medium on methane production and ruminal fermentation in *in vitro* gas production test, using 200 mg of corn meal and Chinese wildrye grass meal mixture in equal proportion as substrate in a 100 m L^{-1} graduated syringe. *In vitro* total gas production, methane concentration and production, pH, ammonia-N, Total Volatile Fatty Acids (TVFA) and protozoa counts were determined after 24 h incubation. Excluding the lower dose concentration (0.15 g L^{-1}) of GSTT *in vitro* methane concentration and production was significantly decreased ($p<0.05$). Compared to control, addition with 0.30, 0.60, 0.90 g L^{-1} of GSTT reduced methane concentration and production by 23.43, 24.93, 25.30% and 26.67, 28.89, 31.11%, respectively. The protozoal counts were decreased by 27.03, 37.84, 60.36 and 72.07%, respectively at levels of 0.15, 0.30, 0.60 and 0.90 g L^{-1} of GSTT compared to that in control. The pH of medium was unaffected by GSTT addition. Ammonia-N concentrations decreased significantly ($p<0.05$) when the higher levels (0.60 and 0.90 g L^{-1}) of GSTT were supplemented. There were no significant differences ($p>0.05$) in TVFA concentrations among levels of GSTT addition. Molar proportion of acetate and butyrate was decreased and propionate was increased with a corresponding reduction in acetate:propionate ratio. In conclusion, these results showed that GSTT has a potential to be used as a feed additive to suppress methane emission.

Key words: *Tribulus terrestris*, saponins, *in vitro* fermentation, methane, volatile fatty acids

INTRODUCTION

In the recent years, there have been growing concerns over methane emissions because of their effects on global warming and climate change (Mirzaei-Aghsaghali and Maheri-Sis, 2011). Typically, methane produced during anaerobic fermentation in the rumen represents 2-12% gross energy loss and emissions from livestock contribute approximately 15% of the total atmospheric methane flux (Moss, 1993; Takahashi, 2011). There is an increasing interest in exploiting natural products as feed additives to decrease methane production for the protection of environment and improving feed conversion efficiency in ruminants (Yurtseven and Ozturk, 2009; Yurtseven *et al*., 2009). Plant extracts containing saponin are of interest as natural feed additives for ruminants because of its positive effects on suppressing methane emission inhibiting rumen protozoa and modulating rumen fermentation patterns (Hristov *et al*., 1999; Pen *et al*., 2006).

Tribulus terrestris is fairly a good source of saponins belonging to plant family of Zygophyllaceae and widely growing in many parts of China. It is regarded as a life-saving fodder for cattle, sheep and goats in the dry areas. *Tribulus terrestris* has adequate crude protein levels and is moderately degradable in the rumen to be utilized by ruminants as a source of nitrogen for microbial protein synthesis (Sebata *et al*., 2005). The Gross Saponins of *Tribulus terrestris* (GSTT) was one of the effective group ingredients which was extracted from the whole plant of caltrop. It is a mixture of >10 kinds of saponins, mainly steroid saponins. Data have been published on its cardiovascular, cytotoxic and antimicrobial activities (Kostova and Dinchev, 2005; Ojha *et al*., 2008) as well as anticancer properties (Neychev *et al*., 2007). However, there is scarce information on the the effects of GSTT on ruminal microbial fermentation. Thus, the aim of the present study was to assess the effects of GSTT on ruminal fermentation characteristics and methane production *in vitro*.

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MATERIALS AND METHODS

The GSTT was purchased from Xi, an Xu-Huang Bio-technology Co., Ltd. According to the manufacturer, content of the saponins was above 900 g kg^{-1}, extracted from the whole plant of caltrop. It mainly includes spiralstrol and furanstrol.

Substrate was a mixture of corn grain (500 g kg^{-1}) and Chinese wildrye (500 g kg^{-1}), thoroughly homogenized and pass through a 1 mm sieve. The substrates contained (g kg^{-1} DM:OM, 970; CP, 84.3; NDF, 428 and ADF, 215). The rumen fluid was collected before the morning feeding from 2 ruminally cannulated, nonlactating Holstein cows (average bodyweight, 600 kg), fed a mixed ration comprising corn stalk silage (600 g kg^{-1}) and concentrate mixture (400 g kg^{-1}) on an as-fed basis at 1.3 times maintenance with free access to clean drinking water. Ruminal contents were collected into a prewarmed insulated flask with an O_2-free headspace, immediately transported to the laboratory, homogenized and squeezed through 4 layers of cheesecloth into a prewarmed Erlenmeyer flask under continuous flushing with CO_2. The two filtrates were combined, on an equal volume basis and used as the source of inoculum.

In vitro gas production was determined by the procedure of Menke and Steingass (1988). Incubations were completed in 100 mL graduated glass syringes in which (200±1 mg) DM of substrate, 10 mL strained rumen fluid and 20 mL incubation medium were dispensed anaerobically and weighed amounts of GSTT were added to achieve final concentrations of 0, 0.15, 0.30, 0.60 and 0.90 g L^{-1} in the culture medium. Each treatment (every dose of GSTT) was incubated in quadruplicate and fermentations were repeated on two separate days. The corresponding blanks consisted of the buffered medium without the substrate but contained GSTT at levels equivalent to that in the test syringes. All syringes were placed in a gentle shaking water bath at 39°C.

The gas produced after 24 h was recorded from visual assessment of the calibrated scale on the syringe. Cumulative gas was expressed as milliliter of gas produced per 200 mg of dry matter and corrected for blanks. A sample of 1 mL of gas was removed from each of the syringes with a gas-tight syringe and analyzed for methane concentration by the Agilent 7890A Gas Chromatograph with a capillary column (30 m×0.32 mm) and flame-ionization detection (Agilent Technologies Inc, USA). The methane values were converted to mmol/g incubated DM. The syringes were uncapped and pH was determined immediately in culture fluid. For ammonia N determination, a 6 mL sample of filtered fermenter fluid was acidified with 2 mL of 25% metaphosphoric acid and frozen. Samples were centrifuged at 12,000×g for 20 min and the supernatant was analyzed by spectro-photometry (UV-120-01, Shimadzu, Kyoto, Japan) for ammonia N (Broderick and Kang, 1980). For analysis of VFA, 1 mL of 25% metaphosphoric acid was added to 5 mL of fermentation fluid, centrifuged (10, 000×g for 10 min at 4°C) and supernatants were stored at -20°C until analyzed by the Agilent 7890A Gas Chromatograph (Agilent Technologies Inc, USA).

About 1 mL of the incubated fluid was diluted with 4 mL of methyl green formalin-saline solution. Protozoa samples were stored at room temperature in darkness until counting. The mixture was pipetted into a refitted counting haemocytometer (depth 0.3 mm). The protozoa were then counted under 10x magnification in a microscope (Ogimoto and Imai, 1981). The data from the experiments were analyzed by a general linear models procedure of SAS Version 9.0 (SAS Institute, 2002). Multiple comparisons among mean values used Duncan's test (Steel and Torrie, 1980). Treatment results are reported as least squares means. The $p<0.05$ was considered as significant.

RESULTS AND DISCUSSION

The addition of GSTT did not influence significantly the *in vitro* total gas production after 24 h incubation. Cumulative total gas production were decreased numerically with the increasing levels of GSTT except 0.15 g L^{-1} (Table 1). Compared to control, addition with 0.30, 0.60 and 0.90 g L^{-1} of GSTT significantly ($p<0.05$) reduced methane concentration by 23.43, 24.93 and 25.30%, respectively. Similarly when expressed as mM per 200 mg of DM, addition with 0.30, 0.60 and 0.90 g L^{-1} of GSTT significantly ($p<0.05$) decreased methane production by 26.67, 28.89 and 31.11%, respectively (Table 1).

The results are in consonance of other reports that associate inhibition of methane production by supplementation of saponin-rich plants such as *Yucca schidigera* extract (Lila *et al*., 2003) and tea saponin (Hu *et al*., 2005). It is possible that the decline in methane emission in the study was attributed to a decrease in protozoal numbers (Table 1). It has been suggested that saponins may decrease methanogen populations through a reduction of numbers of protozoa. Between 9 and 25% of methanogens are associated with ciliate protozoa (Newbold *et al*., 1995), methanogens have both ectosymbionts and endosymbionts with protozoa (Finlay *et al*., 1994).

Rumen protozoa also provide hydrogen as a substrate for methanogens (Stumm and Zwart, 1986). Consequently, methanogen species associated with

Table 1: Effects of gross saponin of Tribulus terrestris on *in vitro* rumen fermentation characteristics at 24 h incubation

	Dose (gL^{-1})				
Items	0	0.15	0.30	0.60	0.90
24 h gas production (mL/200 mg of DM)	46.25±0.50^{ab}	49.13±4.82^{a}	43.75±2.50^{b}	44.00±1.29^{b}	44.50±3.32^{b}
Methane/total gas (%)	24.07±5.21^{a}	20.90±4.71^{a}	18.43±1.35^{b}	18.07±1.06^{b}	17.98±1.73^{b}
24 h CH_4 production (mmol/200 mg of DM)	0.45±0.090^{a}	0.42±0.060^{a}	0.33±0.040^{b}	0.32±0.01^{b}	0.31±0.020^{b}
Protozoal population (1×10^{4} mL^{-1})	22.20±3.71^{a}	16.20±2.64^{b}	13.80±1.77^{b}	8.80±1.72^{c}	6.20±1.640^{c}
pH	6.79±0.060	6.79±0.070	6.80±0.040	6.85±0.09	6.84±0.100
Ammonia-N (mg dL^{-1})	15.79±0.14^{a}	14.82±0.42^{ab}	14.56±0.38^{ab}	13.36±1.87^{bc}	12.85±1.25^{c}
Total VFA ($mmol^{-1}$)	64.62±1.47	63.89±2.83	62.17±1.65	60.98±4.09	60.55±0.96
Acetate (mol 100/mol)	66.19±0.29^{a}	65.81±0.51^{ab}	65.68±0.54^{ab}	64.92±1.54^{ab}	64.69±0.47^{b}
Propionate (mol 100/mol)	22.57±0.26^{a}	22.88±0.58^{a}	23.72±0.57^{ab}	24.65±1.53^{b}	25.00±0.70^{b}
Butyrate (mol 100/mol)	11.24±0.27^{a}	11.31±0.71^{a}	10.60±0.09^{b}	10.43±0.45^{b}	10.30±0.23^{b}
Acetate:propionate	2.93±0.040^{a}	2.88±0.080^{a}	2.77±0.090^{ab}	2.64±0.23^{b}	2.59±0.090^{b}

Values are shown as mean±SD. Values with different letters show statistically significant differences ($p<0.05$)

protozoa usually decrease when protozoal numbers decline (Sharp *et al*., 1998). Additionally, it has been suggested that methane emission may also be affected by saponins as a result of reduced rate of methanogenesis via diminished activity of methane producing gene without changing the total methanogen population (Guo *et al*., 2008). It is also possible that methane was reduced through an inhibition of the growth of H_2-producing bacteria (Wallace *et al*., 1994) including cellulolytic bacteria and other bacteria that use pyruvate ferredoxin oxidoreductase to metabolize pyruvate to acetyl-CoA (Wang *et al*., 2000). Further research is needed to study the effect of GSTT on methanogens and to clarify the mechanism with which the methane emission is inhibited by the GSTT.

Increasing the concentration of GSTT resulted in a significant decrease ($p<0.05$) in total protozoal counts. Total protozoal counts were all lower ($p<0.05$) compared with the control treatment regardless of concentration of GSTT. Similar to these observations many studies have demonstrated that saponins and saponin-containing extracts have inhibitory effects on protozoa (Hu *et al*., 2005; Pen *et al*., 2006; Guo *et al*., 2008). One possible mechanism to explain the effect of saponins on protozoa is a change in cell membrane permeability (Klita *et al*., 1996) as they form complexes with cholesterol in protozoal cell membranes and result in cell lysis. Addition of GSTT did not significantly affect medium pH, all values were in the normal range.

Data revealed that the supplementation of GSTT at the 2 higher levels (0.60 and 0.90 g L^{-1}) decreased ammonia N concentrations significantly ($p<0.05$) as compared to control. According to Williams and Coleman (1991), reduced ammonia-N concentration in the rumen is typical when protozoal counts are inhibited. The reduction in ammonia N levels in the study presumably due to depressed bacterial lysis as a result of anti-protozoal properties of GSTT. Additionally, it is possible that direct inhibition of microbial urease by saponins may lead to reduced ammonia concentrations in the rumen (Ellenberger *et al*., 1985). There were no significant differences ($p>0.05$) in TVFA concentrations among levels of GSTT addition. Similar results have been observed for *Quillaja saponin* (Makkar and Becker, 1996), Yucca extract (Wang *et al*., 1998) and tea saponins (Hu *et al*., 2005). Lila *et al*. (2003) reported an in crease in TVFA concentrations on supplementation of sarsaponins. However, in the study of Pen *et al*. (2006), the feeding of *Quillaja saponaria* extract to sheep reduced TVFA concentrations in the rumen. These results indicate that the nature and dose of saponins affect the TVFA production. Molar proportion of acetate at the high levels of the GSTT treatment (0.90 g L^{-1}) were lower ($p<0.05$) than that of the control treatment. Molar proportion of butyrate was not affected at lower concentration of GSTT (0.15 g L^{-1}) but it was significantly decreased ($p<0.05$) as the concentration of GSTT increased from 0.30-0.90 g L^{-1} after 24 h incubation. Reverse trend was observed for molar proportion of propionate with the significantly ($p<0.05$) higher production at the 2 higher levels of the GSTT treatments (0.60 and 0.90 g L^{-1}) (Table 1).

This tendency is in consonance of Nevel and Demeyer (1996) who reported when methane production is inhibited, a decrease in acetate and a increase in propionate production are expected. Since, propionate production competes with methane for available hydrogen, a increase in propionate production would lead to a concomitant decrease in methane production. Acetate and butyrate are the major endproducts of fermentation in ruminal protozoa (Williams and Coleman, 1997). Consequently, a reduction in protozoal counts by GSTT may result in an increase in the proportions of propionate and a decrease in the acetate:propionate ratio.

CONCLUSION

The results of this experiment indicate that addition of GSTT inhibited the ruminal protozoa numbers,

suppressed methane emission and ammonia-N concentrations and caused a change in the fermentation pattern which was characterized by lower acetate to propionate ratios and higher propionate proportion in *in vitro* batch fermentation. Therefore, GSTT has a potential to be used as a methane inhibitor to manipulate rumen fermentation. Further research is also required to determine the long-term effects on rumen fermentation and animal performance.

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