Open Access
Asian Australas. J. Anim. Sci.
Vol. 27, No. 8 : 1088-1097 August 2014
http://dx.doi.org/10.5713/ajas.2013.13714
www.ajas.info
pISSN 1011-2367 eISSN 1976-5517

Effect of Gynosaponin on Rumen *In vitro* Methanogenesis under Different Forage-Concentrate Ratios

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ABSTRACT: The study aimed to investigate the effects of gynosaponin on *in vitro* methanogenesis under different forage-concentrate ratios (F:C ratios). Experiment was conducted with two kinds of F:C ratios (F:C = 7:3 and F:C = 3:7) and gynosaponin addition (0 mg and 16 mg) in a 2×2 double factorial design. In the presence of gynosaponin, methane production and acetate concentration were significantly decreased, whereas concentration of propionate tended to be increased resulting in a significant reduction ($p<0.05$) of acetate:propionate ratio (A:P ratio), in high-forage substrate. Gynosaponin treatment increased ($p<0.05$) the butyrate concentration in both F:C ratios. Denaturing gradient gel electrophoresis (DGGE) analysis showed there was no apparent shift in the composition of total bacteria, protozoa and methanogens after treated by gynosaponin under both F:C ratios. The real-time polymerase chain reaction (PCR) analysis indicated that variable F:C ratios significantly affected the abundances of *Fibrobacter succinogenes*, *Rumninococcus flavefaciens*, total fungi and counts of protozoa ($p<0.05$), but did not affect the *mcr*A gene copies of methanogens and abundance of total bacteria. Counts of protozoa and abundance of *F.succinogenes* were decreased significantly ($p<0.05$), whereas *mcr*A gene copies of methanogens were decreased slightly ($p<0.10$) in high-forage substrate after treated by gynosaponin. However, gynosaponin treatment under high-concentrate level did not affect the methanogenesis, fermentation characteristics and tested microbes. Accordingly, overall results suggested that gynosaponin supplementation reduced the *in vitro* methanogenesis and improved rumen fermentation under high-forage condition by changing the abundances of related rumen microbes. (**Key Words:** Forage:Concentrate Ratios, Gynosaponin, Methanogenesis, Microbiota, *In vitro*)

INTRODUCTION

Due to the increased concerns of greenhouse gas emissions and the restriction on the use of the antibiotics in the livestock industry, secondary plant metabolites have received noticeable attention as alternative feed additives to modify the rumen microbial ecosystem and suppress enteric methane emission. Saponins are naturally occurring surface-active glycosides, found in various plants in different forms and structures (Vincken et al., 2007). A great many of studies have been conducted to investigate the effects of different saponins on rumen microbial fermentation characteristics and methanogenesis (Lila et al., 2003; Wina et al., 2005; Guo et al., 2008; Pen et al., 2008). However, the results varied depending on the types, doses and structures of saponins, because saponins have different biological activities according to their aglycone portion and combined sugar body of the structure (Hassan et al., 2010). For example, 0.4 g tea saponins (60% saponins) and 7.01 g *Yucca schidigera* extract (8% to 10% saponins) in 1 L culture medium reduced the protozoa numbers by 16.4% and 55.9% and methane production by 14.3% and 41.7% respectively (Hu et al., 2005; Pen et al., 2006), whereas 6.91 g *Quillaja saponaria* extract (5% to 7% saponins) and 0.29 g fenugreek seeds (34% saponins) in 1 L culture medium did not affect the methane production (Pen et al., 2006; Goel et al., 2008a).

Researches have also showed that the effectiveness of saponins on methanogenesis was different depending on the composition of diets. For example, Goel et al. (2008a) noted that methane suppressing effects of saponins from *Sesbenia sesban* and fenugreek were profound in concentrate-based diets compared with roughage based diets. But, Patra et al. (2006) observed that saponins

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Submitted Nov. 12, 2013; Revised Dec. 30, 2013; Accepted Feb. 2, 2014

extracted from *Acacia concinna* did not affect methane production in 1:1 concentrate to roughage based diet.

Gynostemma pentaphyllum Makino, a perennial creeping herb grown prevalently throughout China, India, Japan and Korea (Blumer and Liu, 1999), is a traditional medicine widely used in the treatment of respiratory inflammation such as cough and chronic bronchitis (Tanner et al., 1999). Its medicinal properties have been mainly attributed to the dammarane saponins with 75% being gynosaponin (Kuwahara et al., 1989). As compared with other saponins, gynosaponin has a high saponin content (>98%) and has been showed effective on methane production, fermentation characteristics and cell numbers in fungus-methanogen co-cultures (Wang et al., 2011). However, the effect of gynosaponin on rumen methane production in a rumen system is not known. Thus, the objective of the present i*n vitro* study was, therefore, to evaluate the effects of gynosaponin addition on rumen methanogenesis, fermentation characteristics and microbiota under different forage-concentrate (F:C) ratios.

MATERIALS AND METHODS

Animals and inocula

Three rumen fistulated Nanjing local goats were used for inoculum donor animals. Animals were cared for in accordance with guidelines established by the Chinese Science and Technology Committee Experimental Animal Care and Use guidelines (1998) and the diet was formulated to meet the maintenance requirement (NY/Y 816-2004; Ministry of Agriculture of China, 2004), which included 70% forage (Chinese wildrye) and 30% concentrate mixtures (20% ground corn, 7% soybean meal, 1.5% calcium hydrogen phosphate, 0.5% stone powder, 0.5% sodium chloride, and 0.5% mineral and vitamin premix). Feed was given in equal portions twice daily at 08:00 and 17:00 and the animals had free access to the drinking water. On the day of experiment, rumen contents were collected from three goats just before the morning feeding (0 h) and immediately homogenized and squeezed through two layers of cheesecloth and poured into insulated flasks under anaerobic conditions as described by Zhu et al. (1999) and used as inocula.

Experimental design

Experiment was conducted in a 2×2 double factorial design with substrates Chinese wild rye and concentrate mixture milled to pass through 0.9 mm sieve. The gynosaponin powder, dammarane type triterpene saponin with 98% purity, was from Nanjing Zelang Medical Co. Ltd (China). The culture medium was prepared according to Mao et al. (2007a) using Medium D as defined by Longland et al. (1995). Briefly, micromineral solution 0.1 mL, buffer solution 200 mL, macromineral solution 200 mL, resazurin solution 1 mL and L-cysteine hydrochloride 1 g.

Rumen inocula described above was anaerobically mixed with culture medium, and 60 mL of buffered rumen fluid was then transferred to 160 mL capacity serum bottles that contained 0.6 g total substrate in 7:3 (0.42 g Chinese wild rye, 0.12 g ground corn and 0.06 g soybean meal) and 3:7 (0.18 g Chinese wild rye, 0.28 g ground corn and 0.14 g soybean meal) ratios and with gynosaponin (0 mg and 16 mg). The addition level of gynosaponin was based on previous researches (Hu et al., 2005; Guo et al., 2008; Wang et al., 2011) and the set of serum bottles without gynosaponin was served as control. Each set had four replicates. The serum bottles were sealed with rubber stoppers and aluminum caps and incubated at 39°C for 48 h.

Analytical procedures

Gas and fermentation characteristics: Total gas production was measured at 2, 4, 8, 12, 24, 36, and 48 h of incubation time using a pressure transducer and calibrated syringe (Theodorou et al., 1994). After the total gas measurement, methane production was determined using capillary column gas chromatograph (GC-14B, Shimadzu, Japan), with details as described by Wang et al. (2011). The amount of methane was calculated using standard curves (eight points with triplicate estimations; R^2 coefficients of 0.994). After 48 h incubation, the fermentation was terminated by swirling the bottles on ice and the bottles were opened and the pH of rumen samples was determined immediately using a pH meter (Ecoscan pH 5, Singapore), and then the content samples were homogenized and subpackaged to 5 mL centrifuge tubes for further analyses of fermentation characteristics and microbiota. A portion of 5 mL samples was mixed with freshly prepared 25% meta-phosphoric acid for measuring the volatile fatty acids (VFAs) concentrations using a capillary column gas chromatograph (GC-14B, Shimadzu, Japan), with details as described by Mao et al. (2007a). A portion of 5 mL samples was acidified with 0.5 mL 0.5 mol/L HCl and stored at –20℃ for further analysis of NH_3-N concentration by the indophenols method described by the (Weatherburn, 1967). An aliquot of 5 mL of samples was kept at –20℃ for analysis of ruminal microbial crude protein (MCP) concentrations using a colorimetric method, with 1 mg/mL bovine serum albumin solution (Sigma-Aldrich Co. LLC, St. Louis, Missouri, USA) as a standard equivalent, described by Makkar et al. (1982).

DNA extraction: Total DNA was extracted from culture samples according to the method of Denman and McSweeney (2006). Briefly, cetyltrimethyl ammonium bromide buffer was used and followed by Phenol-Chloroform- Isoamyl alcohol extraction (Zhu et al., 2003) and by using equipment FastPrep-24 (MP Biomedical,

South Florida, USA) and thermomixer compact.

Microbial communities: Rumen microbial communities were revealed by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) technique. Primers used to amplify total bacteria, methanogens and protozoa are shown in Table 1. The PCR was performed using a Taq DNA polymerase Kit (Progma, Madison WI, USA) in a thermocycler (Biometra, Göttingen, Germany). PCR program for bacteria was: 94℃ for 5 min, and 35 cycles of 94℃ for 30 s, 56℃ for 20 s, 68℃ for 40 s, and 68℃ for 7 min last extension; for methanogens was: 94℃ for 1 min; 94℃ for 30 s, 58℃ to 53℃ (0.5℃/cycle), 72℃ for 1 min 10 cycles, 94℃ for 30 s, 56℃ for 30 s and 72℃ for 1 min 25 cycles, 72℃ for 15 min last extension; and for protozoa was: 94℃ for 4 min, 56℃ for 30 s, 72℃ for 1 min, 35 cycles of (56℃ for 30 s, 94℃ for 1 min and 72℃ for 1 min), 94℃ for 4 min; 56℃ for 30 s and 72℃ for 10 min last extension. After checking the sizes and amounts of amplicons using electrophoresis on 1.2% agarose gel containing GoldView, DGGE was performed using a Dcode DGGE system (Bio-Rad, Hercules, California, USA) in 8% polyacrylamide gels containing 37.5:1 acrylamide-biacrylamide and denaturing gradients of 38% to 53%, 40% to 55% and 28% to 43% of urea for bacteria, methanognes and protozoa respectively (Muyzer et al., 1993; Sylvester et al., 2005; Yu et al., 2008). Running progress of electrophoresis, staining, scanning and analyzing the gel electrophoresis profiles were performed by the method as described by Mao et al. (2007b).

Microbial abundances: Samples for protozoa counting were mixed with 9% formaldehyde and preserved at –4℃, then counted in a haemacytometer under light microscope (Nikon YS100, Nikon, Yokohama, Japan) using the method of Dehority (2005). Group-specific primers for methanogens (*mcrA*), total bacteria and anaerobic fungi, and species-specific primers for *R. flavefaciens* and *F. succinogenes* are listed in Table 1, as described by Denman and McSweeney (2006) and Denman et al. (2007). Species of *Methanobrevibacter* sp. (from CSIRO Livestock Industries, St. Lucia, QLD, Australia), rumen anaerobic fungi isolated from rumen contents by Cheng et al. (2006) and *R. flavefaciens* and *F. succinogenes* (from Aberystwyth University, UK) were used to generate standards for real-time PCR analysis. Conventional PCR was performed for respective species and PCR products were quantified using a Nanodrop spectrophotometer ND-1000 UV-Vis (Thermo Fisher Scientific, Inc., Madison, WI, USA). For each standard, copy number concentration was calculated based on the PCR fragment length and the DNA concentration. Real-time quantitative PCR was performed using the ABI 7300 real-time PCR system (Applied Biosystems, Foster, California, USA). Reaction mixture, amplification conditions and performing progresses were conducted by the method of Yang et al. (2012).

Calculation and statistical analysis

Initially, all data were calculated by Microsoft excel software and statistical analysis was carried out using General Linear Model (univariate) procedure of SPSS (version16.0; SPSS Inst. Inc. Cary, NC, USA). When there were statistical changes on the interaction values of treatments, Tukey in the statistical software package SPSS

Table 1. Primers used for DGGE and Real-time PCR

Target organisms	Primers	Sequence (5'→3')	Amplicon (bp)	Reference
Total bacteria	968F-GC	AACGCGAAGAACCTTAC	433	Nübel et al. (1996)
	1401R	CGGTGTGTACAAGACCC		
Methanogens	344F-GC	ACGGGGYGCAGCAGGCGCGA	175	Yu ZT et al. (2008)
	519R	GWATTACCGCGGCKGCTG		
Protozoa	316F	GCTTTCGWTGGTAGTGTATT	223	Sylvester et al. (2005)
	539R-GC	ACTTGCCCTCYAATCGTWCT		
Methanogens	Forward	TTCGGTGGATCDCARAGRGC	140	Denman et al. (2007)
	Reverse	GBARGTCGWAWCCGTAGAATCC		
Total bacteria	Forward	CGGCAACGAGCGCAACCC	130	Denman and McSweeny (2006)
	Reverse	CCATTGTAGCACGTGTGTAGCC		
Fungi	Forward	GAGGAAGTAAAAGTCGTAACAAGGTTTC	120	Denman and McSweeny (2006)
	Reverse	CAAATTCACAAAGGGTAGGATGATT		
R. flavefaciens	Forward	CGAACGGAGATAATTTGAGTTTACTTAGG	132	Denman et al. (2007)
	Reverse	CGGTCTCTGTATGTTATGAGGTATTACC		
F. succinogenes	Forward	GTTCGGAATTACTGGGCGTAAA	121	Denman and McSweeny (2006)
	Reverse	CGCCTGCCCCTGAACTATC		

DGGE, denaturing gradient gel electrophoresis; PCR, polymerase chain reaction.
GC clamp (40 bp) attached to the Primer (CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG).

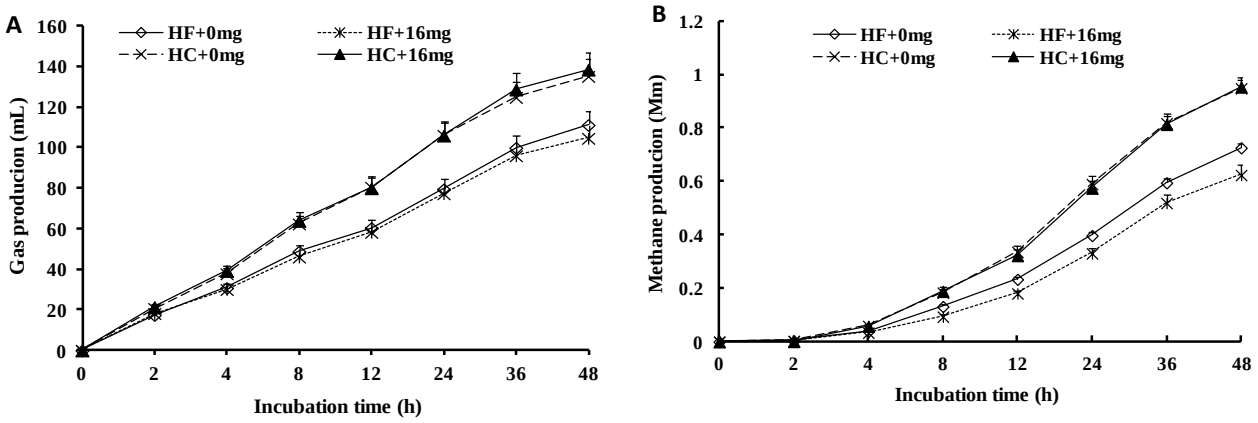

Figure 1. Curves of cumulative gas production (A) and methane production (B). HF+0 mg = high forage (F:C = 70:30)+0 mg gynosaponin; HF+16 mg = high-forage (F:C = 70:30)+16 mg gynosaponin; HC+0 mg = High-concentrate (F:C = 30:70)+0 mg gynosaponin; HC+16 mg = high concentrate (F:C = 30:70)+16 mg gynosaponin. Values presented are the average of the replicate cultures (n = 4). Error bars represent the standard error of the mean.

16.0 was used for further analysis of multiple comparisons of individual treatments.

RESULTS

Total gas, methane production and rumen fermentation characteristics

The cumulative production of total gas and methane during each measuring point are shown in Figure 1A and 1B. The total gas production and methane production showed a similar increasing pattern. However, the total gas production with high-concentrate substrate showed a relatively faster increasing curve than that with high-forage substrate. There was no difference between gyposaponin treatment and the control on cumulative gas production and methane production at each measuring point under high-concentrate substrates. Gynosaponin addition apparently reduced the total gas and methane production from the 4 h of incubation in high-forage substrate. This effect was evident (Table 2) during the 48 h fermentation process, both total gas and methane production of high-concentrate substrate were significantly greater than those of the high-forage substrate. Changes in methane production through gynosaponin treatment were F:C ratio dependent where gynosaponin addition reduced the cumulative methane production by 14.49% ($p<0.05$) in the high-forage substrate after 48 h fermentation, while no changes were observed in high-concentrate level.

The effects of F:C ratio and gynosaponin treatment on fermentation characteristics are also shown in Table 2. As

Table 2. Effects of gynosaponin on *in vitro* rumen 48 h fermentation characteristics at different F:C ratios

Item	Treatment[1] (mg gynosaponin)				SEM	p-value[2]		
	HF+0	HF+16	HC+0	HC+16		F:C	GS	F:C×GS
Gas production (mL)	111.34^{a}	104.76^{b}	135.34^{c}	134.64^{c}	1.80	<0.01	0.05	0.15
Methane (mmol)	0.727^{a}	0.625^{b}	0.950^{c}	0.954^{c}	0.019	<0.01	0.02	<0.01
pH value	6.54^{a}	6.59^{b}	6.36^{c}	6.40^{d}	0.01	<0.01	0.01	0.25
MCP (mg/mL)	0.32	0.32	0.31	0.30	0.00	<0.01	0.13	0.10
NH_3-N (mmol/L)	12.39^{a}	13.23^{a}	16.27^{b}	16.77^{b}	0.98	<0.01	0.51	0.86
TVFA (mmol/L)	74.65^{a}	72.43^{a}	88.81^{b}	91.50^{b}	1.74	<0.01	0.90	0.18
Acetate (mmol/L)	46.54^{a}	41.11^{b}	50.37^{c}	51.40^{c}	1.12	<0.01	0.02	0.01
Propionate (mmol/L)	12.71^{a}	13.75^{b}	14.32^{bc}	14.7^{c}	0.45	0.01	0.14	0.48
Butyrate (mmol/L)	7.91^{a}	9.72^{b}	13.70^{c}	15.12^{c}	0.57	<0.01	0.04	0.74
Valerate (mmol/L)	1.97^{a}	2.05^{a}	3.38^{b}	2.87^{b}	0.25	<0.01	0.42	1.97
Isobutyrate (mmol/L)	2.45^{a}	2.49^{a}	2.97 ^{ab}	3.13^{b}	0.19	0.01	0.60	0.76
Isovalerate (mmol/L)	3.07^{a}	3.32^{a}	4.06^{b}	4.26^{b}	0.18	<0.01	0.23	0.90
A:P ratio	3.70^{a}	2.99^{b}	3.52^{ab}	3.50^{ab}	0.13	0.24	0.02	0.02

F:C ratios, forage-concentrate ratios; SEM, standard error of means; MCP, microbial crude protein; TVFA, total volatile fatty acid.

[1] HF+0 mg = high forage (F:C = 70:30)+0 mg gynosaponin; HF+16 mg = high-forage (F:C = 70:30)+16 mg gynosaponin; HC+0 mg = high-concentrate (F:C = 30:70)+0 mg gynosaponin; HC+16 mg = high concentrate (F:C = 30:70)+16 mg gynosaponin.

[2] F:C = effects of forage concentrate ratios; GS = effects of gynosaponin; F:C×GS = interaction effects of forage concentrate ratios and gynosaponin.

[a,b,c] The means within a row with different superscripts differ significantly ($p<0.05$).

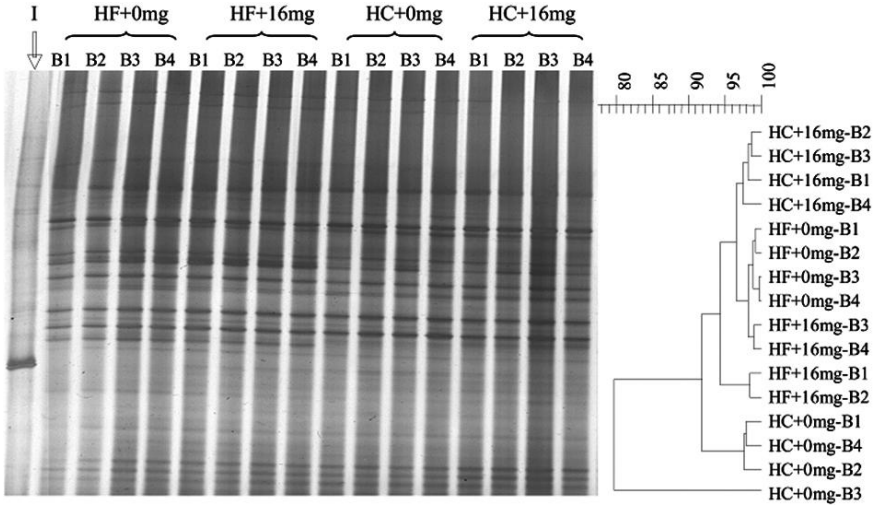

Figure 2. Denaturing gradient gel electrophoresis profile of ruminal bacteria. HF+0 mg = high forage (F:C = 70:30)+0 mg gynosaponin; HF+16 mg = high-forage (F:C = 70:30)+16 mg gynosaponin; HC+0 mg = High-concentrate (F:C = 30:70)+0 mg gynosaponin; HC+16 mg = High concentrate (F:C = 30:70)+16 mg gynosaponin, B1, B2, B3, B4; replications.

compared with the high-forage level, under the high-concentrate condition, the pH value and MCP concentration were lower, whereas concentrations of NH_3-N and the total and individual VFAs were higher. Gynosaponin treatment increased the pH in both high and low F:C ratios, especially significantly under high-forage condition ($p<0.05$), but did not affect the concentrations of NH_3-N and MCP. Under the high-forage condition, the presence of gynosaponin reduced the acetate concentration ($p<0.05$), whereas the propionate concentration slightly increased resulting in a significant reduction of acetate propionate ratio ($p<0.05$). Furthermore, the gynosaponin supplementation increased the butyrate concentrations ($p<0.05$) in both high and low F:C ratios. Except for the butyrate concentration, however, other individual VFA profiles had no changes after treated by gynosaponin under high-concentrate condition.

Rumen microbial communities

Denaturing gradient gel electrophoresis profiles of bacteria, methanogens and protozoa are shown in Figure 2, 3, and 4, respectively. After 48 h fermentation, in addition to some different bands between two kinds of substrates, no special bands appeared for the composition of total bacteria, protozoa and methanogens after treated by gynosaponin in both high and low F:C ratios.

Rumen microbial abundance

Real-time PCR analysis indicated that (Table 3) variable F:C ratios significantly affected abundances of *F. succinogenes*, *R. flavefaciens*, fungi and counts of protozoa,

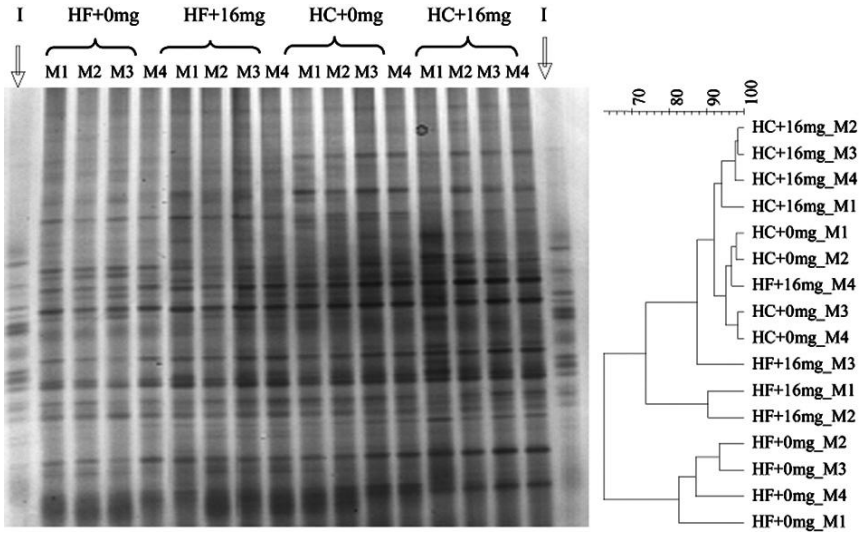

Figure 3. Denaturing gradient gel electrophoresis profile of ruminal methanogens. HF+0 mg = high forage (F:C = 70:30)+0 mg gynosaponin; HF+16 mg = high-forage (F:C = 70:30)+16 mg gynosaponin; HC+0 mg = high-concentrate (F:C = 30:70)+0 mg gynosaponin; HC+16 mg = high concentrate (F:C = 30:70)+16 mg gynosaponin. M1, M2, M3, M4; replications.

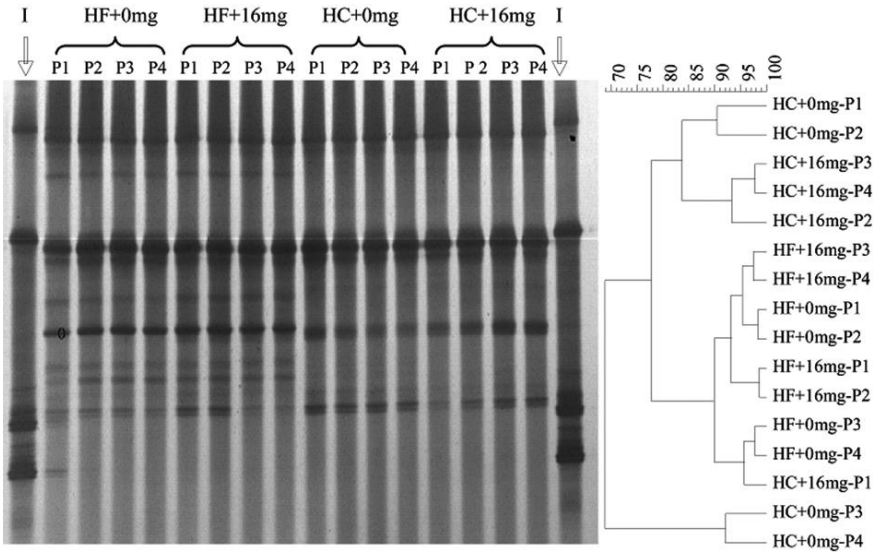

Figure 4. Denaturing gradient gel electrophoresis profile of ruminal protozoa. HF+0 mg = high forage (F:C = 70:30)+0 mg gynosaponin; HF+16 mg = high-forage (F:C = 70:30)+16 mg gynosaponin; HC+0 mg = high-concentrate (F:C = 30:70)+0 mg gynosaponin; HC+16 mg = high concentrate (F:C = 30:70)+16 mg gynosaponin, P1, P2, P3, P4; replications.

but did not affect the *mcr*A gene copies of methangens and abundance of total bacteria. Abundance of *F. succinogenes* and counts of protozoa decreased significantly in high-forage substrate after treated by gynosaponin. The supplementation of gynosaponin also slightly reduced the *mcr*A gene copies of methanogens ($p<0.10$) and abundances of total bacteria, fungi and *R. flavefaciens* under high-forage condition. However, addition of gynosaponin under high-concentrate level did not affect the abundances of above microbes.

DISCUSSION

Total gas production was closely related to the digestion of fermentation substrates and microbial activity and growth. The high total gas production under high-concentrate ratio might be due to the increased activity of related microbes. The reduction of cumulative total gas production with the high-forage substrate during the 48 h fermentation may be because of the reduced individual gases including methane and VFAs.

Studies have suggested that a high-concentrate diet can modulate the rumen fermentation thereby reducing methane emission. For example, Yan et al. (2000) reported the negative correlation between proportion of concentrate in diet and methane emissions. Similarly, Benchaar et al. (2001) demonstrated that methane production was decreased with the replacement of fibrous concentrate with starchy concentrate by 22% and with the utilization of less ruminally degradable starch by 17%. Compared with the forage based diets, feeding concentrate based diets lowers the enteric methane emission (Johnson and Johnson, 1995), since starch fermentation promotes propionate production and lowers ruminal pH, thereby inhibits protozoa and methanogens (Williams and Coleman, 1988). Aguerre et al. (2011) reported that increasing the F:C ratio increased ruminal pH and methane production, but had no effect on manure NH_3-N emission. In our current study however,

Table 3. *In vitro* effects of gynosaponin on rumen microbial population after 48 h incubation at different F:C ratios

Item (copies/mL)	Treatment[1] (mg)				SEM	p-value[2]		
	HF+0	HF+16	HC+0	HC+16		F:C	GS	F:C×GS
Bacteria ($\times10^{10}$)	1.37	1.27	1.23	1.23	0.05	0.08	0.30	0.31
Methanogen ($\times10^{7}$)	9.12	8.40	9.20	9.11	0.22	0.09	0.09	0.17
Fungi ($\times10^{5}$)	2.37^{a}	2.16^{a}	1.83^{b}	1.86^{b}	0.10	<0.01	0.19	0.12
F. succinogenes ($\times10^{7}$)	1.04^{a}	0.86^{b}	0.38^{c}	0.29^{c}	0.05	<0.01	0.02	0.37
R. flavefaciens ($\times10^{7}$)	2.62^{a}	2.49^{a}	1.44^{b}	1.65^{b}	0.11	<0.01	0.76	0.17
Protozoa ($\times10^{3}$)	8.95^{a}	7.85^{b}	6.95^{c}	6.70^{c}	0.27	<0.01	0.03	0.15

F:C ratios, forage-concentrate ratios; SEM, standard error of means.

[1] HF+0 mg = high forage (F:C = 70:30)+0 mg gynosaponin; HF+16 mg = high-forage (F:C = 70:30)+16mg gynosaponin; HC+0 mg = high-concentrate (F:C = 30:70)+0 mg gynosaponin; HC+16 mg = high concentrate (F:C = 30:70)+16 mg gynosaponin.

[3] F:C = effects of forage concentrate ratios; GS = effects of gynosaponin; F:C×GS = interaction effects of forage concentrate ratios and gynosaponin.

[a,b,c] The means within a row with different superscripts differ significantly ($p<0.05$).

under the high-concentrate level the total gas and methane emission was greater than the high-forage condition. The most likely reason for this was high-concentrate conditions can offer more fermentable substrates for bacteria and methanogens, thus producing more H_2 and or formic acid and acetate, leading to more methane, since H_2, formic acid and acetate are effective methanogenic substrates (Bauchop and Mountfort, 1981). In addition, *in vitro* studies though valuable, may not reflect the real activity of diets in the rumen. Indeed, total and individual VFAs were increased with high-concentrate level as compared with high-forage level (Table 2).

The addition of gynosaponin showed no effect on methane production under high concentrate conditions. However, supplementation of gynosaponin inhibited methane production by14.49% under high-forage condition. A great many of *in vitro* researches were conducted to investigate the effects of various saponins on rumen methane production under different F:C ratios. Holtshausen et al. (2009) noted that during *in vitro* 24 h fermentation *Quillaja saponaria* 0.75 g/L in forage based (F:C = 51:49) substrate decreased the methane production by 11.4% and Lila et al. (2003) observed that sarsaponin 1.2 g/L in forage based (F:C = 1.5:1) substrate decreased the methane production by 13.5%. However, Pen et al. (2006) reported that *Quillaja saponaria* extract 2.3 g/L in F:C = 1:1 substrate did not affect methane production. Goel et al. (2008a) demonstrated that methane suppressing effects of saponins from *Sesbenia sesban* and fenugreek were pronounced in concentrate-based diets comparing with roughage based diets. All inconsistencies among above the studies about effects of saponins on methane production may have been due to many factors including the types, doses and structures of saponins and composition of diets (Beauchemin et al., 2008).

Microbial protein, pH value, ammonia-nitrogen and variable VFAs are important rumen inner environmental parameters. In this study, gynosaponin addition increased the pH value under both high and low F:C ratios, especially under high-forage level, but did not affect the MCP and NH_3-N concentrations. The effects of saponins on rumen pH values were various, with some studies showing a reduction (Goetsch and Owens, 1985; Wu et al., 1994), while other studies showed an increase (Wang et al., 2011) or no effect (Hussain and Cheeke, 1995; Hristov et al., 1999) after treatment with saponins. Consistent with our study, Hristov et al. (1999) reported that *Yucca schidigera* extract had no significant effect on microbial protein and Mao et al. (2010) reported that amino-nitrogen concentration was little affected by tea saponin addition.

Coupled with the change of methane emission, our results showed that under a high-forage condition, gynosaponin addition significantly reduced acetate concentration, while slightly increased propionate proportion, thereby resulting in a significant reduction of A:P ratio. This was in agreement with *in vitro* observations of Wina et al. (2005) and Staerfl et al. (2010) that the acetate proportion was reduced significantly by *Yucca* saponins and methanol extract of *Sapindus rarak* addition respectively. Wang et al. (2011) also found the reduction of acetate concentration after treated by gynosaponin in a fungus-methanogen co-culture system. In addition, consistent with our results, increased propionate concentrations and considerably reduced A:P ratios appeared to be achieved in several *in vitro* (Lila et al., 2003; Wina et al., 2005; Pen et al., 2006; 2008) studies. Patra et al. (2010) concluded that saponins might increase the propionate production as a result of rechannelling of hydrogen from methane to propionate and decrease the A:P ratio, which is nutritionally beneficial for ruminants. Mao et al. (2010) reported that methane-suppressing effects of saponins could lead to hydrogen accumulation. However, the high partial pressure of hydrogen and high $NADH/NAD^+$ ratio in the rumen due to the inhibition of methanogenesis may result in a decrease of acetate production (Miller, 1995). Except for the above change in VFA proportions, a slight increase of total VFA concentration was also observed related to the gynosaponin addition under high-forage substrate. Accordingly, the increased pH value in our study might be due to the reduction of the VFAs concentrations in high-forage level. In addition, increased butyrate concentration of treatment groups in both high and low F:C ratios were found in this experiment. But, the mechanism causing the increased butyrate concentration after treated by gynosaponin will need further research.

With DGGE patterns, there were some different bands between two kind substrates, but no special bands appeared for the composition of total bacteria, protozoa and methanogens after treated by gynosaponin in both F:C ratios. This may suggest that gynosaponin treatment did not have an affect the microbiota in the rumen. Possibly due to sufficient fibrous materials offered for the fiber degrading microbes, the abundances of *F. succinogens*, *R. flavefaciens*, fungi and counts of protozoa were significantly increased under high-forage conditions as compared with the high-concentrate substrates. Inclusion of gynosaponin in high-forage substrate significantly reduced the counts of protozoa and abundance of *F. succinogenes*, also slightly reduced the *mcr*A gene copies of methanogens and abundances of total bacteria, fungi and *R. flavefaciens.* However, addition of gynosaponin to the high-concentrate level did not affect the abundances of above microbes.

Most studies (Moss et al., 2000; Hu et al., 2005; Goel et al., 2008b) found the methane inhibitory effects of variable

saponins and these authors contributed this to reduction in protozoa numbers, since protozoa have a symbiotic association with methanogens (Finlay et al., 1994) and methanogens associated with protozoa accounted for as much as 37% decreased methane production (Bhatta et al., 2009). A strong association of protozoal number and methanogenesis was evident in this experiment and this association seemed to be F:C dependent. Gynosaponin substantially reduced the protozoa numbers and methane production in high-forage substrate, but showed little effect on methane production with high-concentrate substrate. In high-concentrate substrate, protozoa counts were significantly lower than the high-forage condition, thus gynosaponin addition to high-concentrate substrate may not show evident effects on protozoa. In the high-forage substrate however, gynosaponin inhibited the protozoa counts. Thus, it is possible that when protozoa counts were higher, the effect of saponins would be more profound.

Indeed, in our present study methanogens, fungi, *F. succinogenes* and *R.flavefaciens* were less abundant after treated by gynosaponin under high-forage substrate. Several *in vitro* studies have reported the direct inhibitory effects of saponins on methanogens. As Goel et al. (2008b) and Wang et al. (2011) noted that saponins significantly reduced methane concentration and inhibited the methanogen growth. Accordant with our results, Wina et al. (2005) observed the toxicity of feeding saponins at high levels occurred to protozoa, fungi and bacteria. Wang et al. (2011) reported that growth of the fungus was decreased by high level gynosaponin addition. Wang et al. (2000) demonstrated that abundances of *F. succinogenes* and *R. flavefaciens* were negatively affected by high level of saponins. Lu and Jorgensen (1987) and Wang et al. (2000) reported that fiber degrading bacteria were more sensitive to saponins than starch degrading bacteria. This may partly explain the current finding that the fibre-degrading bacterial species were evidently affected while the total bacterial population was less affected.

Since Wallace et al. (1994) reported that purified saponins were toxic to rumen microorganisms, the inhibitory effect of gynosaponin on tested microorganisms could be due to its high saponin content as the gynosaponin used in our experiment contained 98% high saponin purity. Furthermore, Saponins have sterol-binding capability in protozoa and fungi cell membranes (Bodas et al., 2008). Wang et al. (2012) demonstrated that expression of *mcr*A gene is closely related to the activity of methanogens and methane production. Thus, the slight reduction of *mcr*A gene copies of methanogens in our study indicates a lowered activity of methanogens. However, the mechanism behind the reduction of methanogens related to saponins is still not yet clear. Further research is needed to elucidate the underlying mechanism.

CONCLUSION

Gynosaponin supplementation inhibited the rumen *in vitro* methane production in high-forage conditions by changing fermentation characteristics and the abundance of related microbes.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (31072052) and China (MOST) – Australia (IIST) joint project (2010DFA31040). The authors thank Dr Christopher S. McSweeney (CSIRO Livestock Industries, Australia) for providing *Methanobrevibacter* and Dr Eun Joong Kim (Aberystwyth University, UK) for *R. flavefaciens* and *F. succinogenes*.

CONFLICTS OF INTEREST

Bakhetgul Manatbay conducted animal experiment, performed DGGE and q-PCR, analyzed the data and drafted the manuscript. Yanfen Cheng helped with methane measurement, DGGE and q-PCR. Shengyong Mao helped with experiment design and statistical analysis. Weiyun Zhu conceived this study, finalized the manuscript and revised the manuscript. We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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