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Effects of phytogenic products on *in vitro* rumen fermentation and methane emission in goats*

G.Z. Dong[1], X.J. Wang, Z.B. Liu and F. Wang

College of Animal Science and Technology, Southwest University
Key Laboratory of Grass and Herbivores of Chongqing
Beibei, Chongqing, 400716, P.R. China

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ABSTRACT

This study evaluated the effects of addition of lucerne extract (LE), *Artemisiae annuae* extract (AAE), and mixed herbal medicine (MHM) into different goat diets on *in vitro* rumen fermentation and methane production. In addition to the negative control (NC), addition of monensin (MO) served as the positive control (PC). Four ruminally cannulated Nanjiang Yellow goats (45±2 kg liveweight) were used as donors of ruminal fluid. The results showed: 1. compared with NC, addition of all the additives into the mixed grass diet increased propionate concentration ($P<0.05$). Protozoa numbers and methane production were reduced by addition of all the additives ($P<0.05$); 2. compared with NC, LE and AAE in the lucerne diet increased propionate concentration ($P<0.05$). All the additives reduced protozoa numbers ($P<0.05$). Methane production was decreased by addition of LE compared with NC and PC ($P<0.05$); 3. addition of LE into the mixed grass-concentrate diet resulted in an increase of the propionate concentration compared with NC and PC ($P<0.05$). Protozoa numbers were reduced by addition of LE and AAE compared with NC ($P<0.05$). Methane production was decreased by addition of AAE compared with NC and PC ($P<0.05$); 4. compared with NC, addition of AAE and MHM into the lucerne-concentrate diet resulted in an increase of propionate concentration ($P<0.05$). Protozoa numbers were reduced by addition of all the additives ($P<0.05$). Methane production was decreased by addition of AAE and MHM ($P<0.05$).

In conclusion, addition of LE, AAE and MHM into different diets reduced methane production, increased propionate concentration and decreased protozoa numbers to a certain extent, and the inhibitory effects of the phytogenic products on methane production are more remarkable in the mixed-grass diet. The phytogenic products appear to be promising alternatives to MO in altering *in vitro* rumen fermentation and reducing methane production in goats.

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[1] Corresponding author: e-mail: gzdong@swu.edu.cn

KEY WORDS: lucerne extract, *Artemisiae annuae* extract, herbal medicine, rumen fermentation, methane, goats

INTRODUCTION

Ruminal methane production represents a loss of 2 to 15% dietary energy in ruminant animals and is also a major contributor to greenhouse effect (Holter and Young, 1992; Johnson and Johnson, 1995). Reducing ruminal methane production could improve animal performance and decrease greenhouse effect. Monensin (MO) and other ionophores have been used to effectively manipulate ruminal fermentation and mitigate ruminal methane production (Spiers, 1992; Nagaraja, 1995; Sauer et al., 1998; García-González, 2008a,b). However, nowadays the public are more concerned about the use of antibiotics which may lead to health problems. Phytogenic feed additives are plant-derived products and are well-known to exert antimicrobial actions (Windisch et al., 2008). Previous studies showed some plant extracts (Hess et al., 2003; Pen et al., 2006) and some plant species (*Rheum officinale*; García-González et al., 2008a,b) were potential alternatives to antibiotics in manipulating rumen fermentation and reducing ruminal methane production. Lucerne extract (LE), *Artemisiae annuae* extract (AAE) and herbal medicines (HM) are phytogenic products available in many parts of the world. Nonetheless, the use of LE, AAE and HM to alter rumen fermentation pattern to reduce methane production was not reported to our knowledge, and studies on the effects of LE, AAE and HM on rumen fermentation and methane production in goats are lacking. Goats are widely-raised livestock in Asia and other parts of the world, so methane emission in goat production should not be underestimated. It was hypothesized in this study that LE, AAE and HM could change the rumen fermentation pattern to reduce methane production in goats. Therefore, the objective of the present study was to evaluate the effects of dietary addition of LE, AAE, and HM with reference to MO on *in vitro* rumen fermentation and methane production in goats.

MATERIAL AND METHODS

Ruminal fluid donor goats and their diet

Four ruminally cannulated Nanjiang Yellow goats (45±2 kg liveweight) were used as donors of ruminal fluid and were individually penned indoors. The use and care of the goats were in accordance with the Regulation on the Care of Experimental Animals issued by the Science and Technology Commission of

Chongqing Municipality. The goats were fed daily 1 kg (dry matter basis) diet (Table 1) twice at 08.00 and 17.00 h and had free access to water at all time.

Table 1. Composition and nutritive value of the diet for ruminal fluid donor goats

Item	%
Ingredients, %	
rice straw	30.80
ryegrass hay	16.84
maize	43.38
rapeseed meal	5.29
dicalcium phosphate	1.54
limestone	0.77
salt	0.38
premix[1]	1.00
Nutrient level[2]	
metabolizable energy, MJ/kg	8.72
crude protein, %	9.1
crude fibre, %	12.9
NDF, %	33.9
ADF, %	22.9
Ca, %	0.88
P, %	0.59
Ca/P	1.49

[1] provided per kg of premix: mg: Fe (as ferrous sulphate) 4000, Cu (as sulphate) 2000, Zn (as sulphate) 8000, Mn (as sulphate) 12000, I (as iodate) 50, Se (as selenite) 10, Co (as chloride) 18, IU: vit. A 90000, vit. D_3 15000, vit. E 3000

[2] analysed values except for ME

Fermentation diets and additives

In this study, three additives were added into 4 types of diet, respectively. The diet types were: 1. mixed grass hay (harvested in spring and consisting of *Alopecurus aequalis Sobol.* 37.9%, *Stellaria media* (L.) Cyr. 35.1% and *Avena fatua* L. 27.0%), 2. lucerne hay, 3. mixed grass-concentrate diet, 4. lucerne-concentrate diet (Table 2). The additives include LE (added at 10 g/kg diet and provided by the Key Laboratory of Grass and Herbivores of Chongqing, Southwest University, China), AAE (added at 10 g/kg diet and provided by the Key Laboratory of Grass and Herbivores of Chongqing, Southwest University, China), and mixed HM (MHM, added at 60 g/kg diet and consisting of *Dryopteris crassirhizoma Nakai* 20.4%, massa fermentata 25.4%, *Astragalus membranaceus* (Fisch.) Bge. 16.9%, *Crataegus pinnatifida* Bge. 16.9%, *Mentha haplocalyx* Briq. 20.4%).

Table 2. Composition and nutritive value of the *in vitro* fermentation diets

Item	Mixed grass-concentrate	Lucerne-concentrate
Ingredients, %		
mixed grass hay	56.00	-
lucerne hay	-	56.00
maize	37.55	37.55
rapeseed meal	3.20	3.20
dicalcium phosphate	1.50	1.50
limestone	0.50	0.50
salt	0.25	0.25
premix[1]	1.00	1.00
Nutrient level[2]		
metabolizable energy, MJ/kg	10.37	10.65
crude protein, %	9.3	14.3
crude fibre, %	15.9	14.7
NDF, %	35.6	30.1
ADF, %	20.9	23.1
Ca, %	0.94	1.01
P, %	0.65	0.62
Ca/P	1.44	1.63

[1] provided per kg of premix: mg: Fe (as ferrous sulphate) 4000, Cu (as sulphate) 2000, Zn (as sulphate) 8000, Mn (as sulphate) 12000, I (as iodate) 50, Se (as selenite) 10, Co (as chloride) 18; IU: vit. A 90000, vit. D_3 15000, vit. E 3000; [2] analysed values except for ME

There were two controls for each type of diet, i.e. the negative control (without additives, NC) and the positive control (MO added at 15 mg/kg diet, PC).

In vitro incubations

The rumen fluid from the 4 donor goats was collected before the morning feeding, immediately mixed and strained into a triangular flask which was placed in a water bath (39°C) and was bubbled with CO_2 continuously. For each incubation (8 replicates per incubation), 200 mg of the diets with different additives was accurately weighed and placed into the fermentation syringes, and the syringes were filled with 30 ml of a medium consisting of 10 ml of the rumen fluid and 20 ml of buffer solution. The medium was prepared as described by Menke and Steingass (1988). The syringes were placed in an air bath (39°C) which was shaken gently, and the incubation continued for 24 h.

Assay methods

The total gas production from the fermentation was measured from the scales of graduated syringes. A sample of 10 ml gas from inside the syringe was used for measuring methane concentration. After the plunger was removed, a sample

of the fermentation end product was taken for measuring pH values immediately using a pH meter. The remaining end product was at once collected and stored in a refrigerator at 4°C for termination of the fermentation.

Methane concentration in the gas was determined by gas chromatography (GC 2100) as descried by Zhang (2006). The chromatographic conditions were set as follows: column (TDX-01) temperature, 80°C; pressure, 130 kpa; total flow, 27.7 m1/min; column flow, 1.55 m1/min; linear velocity, 38.2 m1/min; purge flow, 3.0 m1/min; split ratio, 15.0. Methane production was calculated by multiplying gas production and methane concentration in the gas.

Concentration of NH_3-N in the fermentation end product were determined according to Chaney and Marbach (1962). A sample (0.5 ml) of the fermentation end product was mixed with 2 ml methylgreen-formaldehyde-saline solution and shaken gently for 5 min. It was then pipetted into a refitted haemocytometer (0.44 mm in depth) and protozoa were counted with microscopy according to Lu and Xie (1990).

Volatile fatty acids concentrations in the fermentation end product were determined using gas chromatography (GC 2100) equipped with a H_2-flame detector, and the column used was HP-INNOWAX (19091N-133) with strong polarity. The protocol was in accordance with that of Zhang (2006). The chromatographic condition was as follows: column temperature, 170°C; pressure, 90.1 kpa; total flow, 38.7 m1/min; column flow, 0.70 m1/min; linear velocity, 23.8 m1/min; purge flow, 3.0 m1/min; split ratio, 50.0.

Statistical analyses

Data of all treatments were analysed by one-way analysis of variance, and the differences among means of treatments were tested for significance using q test. All data analyses were performed using the SPSS (13.0) software.

RESULTS

Compared with NC, addition of all the additives into the mixed grass diet increased propionate concentration ($P<0.05$) (Table 3). Supplementation of all the additives reduced butyrate concentration ($P<0.05$). The acetate:propionate ratio was decreased due to the addition of MHM ($P<0.05$). Supplementation of all the additives reduced protozoa numbers ($P<0.05$). Methane production was decreased by supplementation of all the additives ($P<0.05$). Addition of the additives had no effects on pH, NH_3-N and total gas production ($P>0.05$).

Table 3. Addition of lucerne extract (LE), *Artemisiae annuae* extract (AAE) and mixed herbal medicine (MHM) into the mixed grass diet on *in vitro* rumen fermentation and methane emission

Item	NC[1]	PC[2]	LE	AAE	MHM	SEM	P values
Acetate, mmol/l	53.2	56.4	54.2	56.1	51.7	6.10	0.453
Propionate, mmol/l	26.4[a]	29.1[b]	29.9[b]	29.7[b]	32.3[b]	3.42	0.045
Butyrate, mmol/l	21.2[a]	14.1[b]	16.0[b]	14.8[b]	16.1[b]	1.91	0.056
Acetate:propionate	2.01[a]	1.93[a]	1.81[a]	1.88[a]	1.60[b]	0.146	0.032
pH	6.93	6.95	6.94	6.95	6.92	0.623	0.862
NH_3-N, mmol/l	16.79	17.03	17.44	17.63	16.91	3.312	0.543
Protozoa, $\times 10^5$/ml	1.614[a]	1.124[b]	1.113[b]	0.921[b]	1.120[b]	0.335	0.032
Gas production, ml	41.09	40.02	43.45	44.32	43.52	7.764	0.653
Methane content, %	17.12[a]	13.01[b]	13.68[b]	12.12[b]	11.12[b]	2.765	0.036
Methane emission, ml	7.05[a]	5.21[b]	5.90[b]	5.32[b]	4.84[b]	1.064	0.038

[1] negative control; [2] positive control, monensin added at 15 mg/kg; [a,b] means without a common superscript within the same row differ significantly (P<0.05)

Compared with NC, LE and AAE in the lucerne diet increased propionate concentration (P<0.05) (Table 4). Butyrate concentrations were decreased by supplementation of all the additives (P<0.05). Addition of AAE lowered the acetate:propionate ratio (P<0.05). All the additives reduced protozoa numbers (P<0.05). Methane production was decreased by addition of LE compared with both NC and PC (P<0.05). Supplementation of the additives did not affect pH, NH_3-N and total gas production (P>0.05).

Table 4. Addition of lucerne extract (LE), *Artemisiae annuae* extract (AAE) and mixed herbal medicine (MHM) into the lucerne diet on *in vitro* rumen fermentation and methane emission

Item	NC[1]	PC[2]	LE	AAE	MHM	SEM	P values
Acetate, mmol/l	52.7	58.7	54.6	51.2	49.4	6.73	0.553
Propionate, mmol/l	22.5[a]	29.7[b]	27.1[b]	31.0[b]	20.1[a]	4.51	0.057
Butyrate, mmol/l	21.4[a]	17.5[b]	17.8[b]	14.9[b]	16.9[b]	2.30	0.078
Acetate:propionate	2.34[a]	1.98[a,b]	2.01[a]	1.65[b]	2.46[a]	0.234	0.045
pH	6.88	6.83	6.92	6.83	6.85	0.712	0.764
NH_3-N, mmol/l	19.67	19.75	19.37	19.56	19.65	3.678	0.672
Protozoa, $\times 10^5$/ml	2.811[a]	1.654[b]	2.011[b]	1.740[b]	1.823[b]	0.324	0.045
Gas production, ml	51.65	50.02	49.77	49.59	53.04	8.123	0.843
Methane content, %	18.56[a]	17.78[a]	15.62[b]	18.56[a]	18.76[a]	2.998	0.048
Methane emission, ml	9.59[a]	8.90[a]	7.74[b]	9.20[a]	9.95[a]	1.443	0.040

[1,2] see Table 3 for explanation; [a,b] means without a common superscript within the same row differ significantly (P<0.05)

Addition of LE into the mixed grass-concentrate diet resulted in an increase of the propionate concentration compared with both NC and PC (P<0.05) (Table 5). Butyrate concentrations were decreased by addition of AAE and MHM compared with NC (P<0.05). Supplementation of the additives did not affect the acetate:propionate ratio compared with both NC and PC (P<0.05). Protozoa numbers were reduced by addition of LE and AAE compared with NC (P<0.05).

Table 5. Addition of lucerne extract (LE), *Artemisiae annuae* extract (AAE) and mixed herbal medicine (MHM) into the mixed grass-concentrate diet on *in vitro* rumen fermentation and methane emission

Item	NC[1]	PC[2]	LE	AAE	MHM	SEM	P values
Acetate, mmol/l	52.6	55.6	54.6	56.9	56.1	5.94	0.621
Propionate, mmol/l	28.5[a]	29.8[a]	31.1[b]	28.4[a]	27.7[a]	2.92	0.065
Butyrate, mmol/l	17.1[a]	14.6[b]	16.7[a]	14.1[b]	12.5[b]	2.11	0.058
Acetate:propionate	1.85	1.87	1.75	2.00	2.01	0.201	0.055
pH	6.96	7.00	6.96	6.95	6.96	0.812	0.923
NH_3-N, mmol/l	16.65	17.53	17.40	17.09	16.89	4.549	0.823
Protozoa, $\times 10^5$/ml	1.235[a]	0.783[b]	0.839[b]	0.690[b]	1.076[a]	0.212	0.038
Gas production, ml	50.56	52.13	53.33	52.71	54.01	9.102	0.912
Methane content, %	13.78[a]	12.01[a]	13.32[a]	9.53[b]	12.75[a]	3.011	0.039
Methane emission, ml	6.98[a]	6.26[a]	7.09[a]	5.01[b]	6.85[a]	1.424	0.044

[1,2] see Table 3 for explanation; [a,b] means without a common superscript within the same row differ significantly (P<0.05)

Methane production was decreased by supplementation of AAE compared with both controls (P<0.05). pH, NH_3-N and total gas production were not affected by supplementation of the additives (P>0.05).

Compared with NC, addition of AAE and MHM into the lucerne-concentrate diet resulted in an increase of propionate concentration (P<0.05) (Table 6).

Table 6. Addition of lucerne extract (LE), *Artemisiae annuae* extract (AAE) and mixed herbal medicine (MHM) into the lucerne-concentrate diet on *in vitro* rumen fermentation and methane emission

Item	NC[1]	PC[2]	LE	AAE	MHM	SEM	P values
Acetate, mmol/l	59.3	57.5	58.6	54.5	57.5	6.13	0.762
Propionate, mmol/l	27.5[a]	31.1[b]	28.3[a]	33.1[b]	31.0[b]	3.14	0.076
Butyrate, mmol/l	16.7[a]	14.6[b]	16.9[a]	17.0[a]	15.3[a,b]	2.32	0.089
Acetate:propionate	2.15[a]	1.85[a,b]	2.07[a]	1.65[b]	1.85[a,b]	0.201	0.051
pH	6.89	6.84	6.87	6.83	6.87	0.989	0.878
NH_3-N, mmol/l	21.07	20.96	20.89	20.99	20.91	7.587	0.654
Protozoa, $\times 10^5$/ml	2.012[a]	1.631[b]	1.764[b]	1.560[b]	1.682[b]	0.322	0.032
Gas production, ml	57.46	55.68	55.01	56.77	60.41	10.011	0.881
Methane content, %	21.53[a]	15.72[b]	22.61[a]	13.12[b]	15.45[b]	4.676	0.054
Methane emission, ml	12.38[a]	8.75[b]	12.43[a]	7.45[b]	9.33[b]	2.075	0.051

[1,2] see Table 3 for explanation; [a,b] means without a common superscript within the same row differ significantly (P<0.05)

Butyrate concentrations were not affected by supplementation of the additives. The acetate:propionate ratio was lowered by addition of AAE (P<0.05). Protozoa numbers were reduced by addition of all the additives (P<0.05). Methane production was decreased by addition of AAE and MHM (P<0.05). Supplementation of the additives had no effects on pH, NH_3-N and total gas production (P>0.05).

DISCUSSION

In this study, MO had a constant effect on *in vitro* rumen fermentation and methane reduction irrespective of the diet type. This result is consistent with those of other studies (O'Kelly and Spiers, 1992; Nagaraja, 1995; Sauer et al., 1998; García-González, 2008b). When methane production is inhibited, a decrease in both acetate and butyrate and a concomitant increase in propionate production are expected (Van Nevel and Demeyer, 1992).

MO directly inhibits hydrogen-producing bacteria, i.e. acetate and butyrate producing bacteria (Chen and Wolin, 1979; Russell and Houlihan, 2003), which causes a decrease in methane production due to the shortage of molecular hydrogen. MO also favours propionate producing bacteria (Jalc et al., 1992; Newbold et al., 1993), which results in an increase in propionate production. Many studies including both *in vitro* and *in vivo* trials (O'Kelly and Spiers, 1992; Nagaraja, 1995; Sauer et al., 1998; García-González, 2008a,b) suggested that addition of MO reduced methane production, decreased acetate content and the acetate:propionate ratio, increased propionate content, and inhibited protozoa proliferation. Our results are in concordance with those of previous studies.

The effects of LE, AAE and MHM were similar to some extent with those of MO in this study. Supplementation of these phytogenic products reduced by and large methane production. Since methane production is inhibited, a decrease in both acetate and butyrate and a concomitant increase in propionate production were observed. This is in agreement with that reported recently by García-González (2008a). It is noteworthy that all of the phytogenic products caused significant reduction of methane production in the mixed grass diet, whereas only one or two of the phytogenic products exhibited inhibitory effects on methane production in other types of diet. Therefore, the inhibitory effects of the phytogenic products on methane production appear to be diet type dependent, which may have been due to different dietary nutrient composition and balance. It seems that the inhibitory effects of the phytogenic products on methane production are lessened in more nutritionally-balanced diets. According to a recent study of Christophersen et al. (2008) and previous observations (Van Kessel and Russel, 1996; Baker, 1997; Russell, 1998), when the dietary nutrient composition was changed through inclusion of concentrate, the fermentation pattern was altered, which favoured the production of propionate over methane.

Some plant species contain secondary metabolites which may effect on the populations of microorganisms in the rumen and thus change the ruminal fermentation pattern. The chemical compositions of phytogenic products are complicated, with phenolic compounds being the principal active components (Burt, 2004). The antimicrobial mode of action of phytogenic products remains

unclear. Windisch et al. (2008) have pointed out the antimicrobial mode of action of phytogenic products arises mainly from the potential of the phenolic essential oils which intrude into the bacterial cell membrane, disintegrate membrane structures, and cause ion leakage. Essential oils exerted a direct effect on bacterial cell membranes due to their hydrophobic nature and lipophilic character, so they had a high affinity for lipids of bacterial cell membranes (Benchaar et al., 2008). It was reported that essential oils (Busquet et al., 2005a,b) decreased methane production. Antibacterial activities of phytogenic products were also reported from a variety of nonphenolic substances (Windisch et al., 2008). Studies showed saponins decreased methane production (Hess et al., 2003; Hu et al., 2005). Saponins also have anti-protozoal effects (Hu et al., 2005). One possible mechanism to explain the effect of saponins on protozoa is that saponins can change cell membrane permeability (Klita et al., 1996). Hess et al. (2003) pointed out that, of all rumen microbes, protozoa were particularly susceptible to saponin-induced changes in cell membrane properties. In our present study, all phytogenic additives (LE, AAE and MHM) caused a decrease in protozoa numbers. Patra et al. (2006) also reported that the extracts of *Acacia concinna*, *Azadirachta indica* and *Terminalia chebula* reduced total protozoa counts significantly. Since protozoa produce a large amount of hydrogen, methanogens are attached to the surface of protozoa (Lee et al., 2000) to utilize hydrogen. The reduction of protozoa numbers, therefore, favours a decrease in methane production. On the other hand, propionate is an end product of fermentation that requires hydrogen for synthesis. So the lowered methane production resulting from reduced protozoa numbers would be conducive to higher propionate production and lower acetate and butyrate yield observed in this study.

In this study, the additives had no effects on pH, NH_3-N and total gas production. Therefore, based on the results of this study, the rumen pH and nitrogen metabolism profiles were not adversely affected by supplementation of the phytogenic products. Since there exists a highly positive correlation between total gas production and feed degradability, higher total gas production represents to a certain extent higher feed digestibility (Grant and Mertens, 1992; Cone et al., 1996; Blummel et al., 1997). Thus, the addition of the additives might not adversely affect the digestibility of the diets. However, Patra et al. (2006) cautioned that *in vitro* dry matter and organic matter digestibilities of feed were decreased significantly with the addition of several plant extracts, such as the extracts of pods of *Acacia concinna* (Shikakai), seed pulp of *Terminalia chebula* (harad), *Terminalia belerica* (bahera), *Emblica officinalis* (amla) and seed kernel of *Azadirachta indica* (neem seed). This inconsistency could be attributed to different plant species, the amount of extracts supplemented, and the ruminal fluid donor animals.

CONCLUSIONS

Based on the present study, addition of lucerne extract, *Artemisiae annuae* extract and herbal medicines mixture into different diets reduced methane production, increased propionate concentrations and decreased protozoa numbers to a certain extent. The inhibitory effects of the phytogenic products on methane production are more remarkable in the mixed-grass diet. The phytogenic products involved in the study appear to be promising additives and alternatives to monensin in altering rumen fermentation pattern to reduce methane production in goats. However, further validation with *in vivo* studies is warranted.

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