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Effect of Addition of Three Plant Extracts on Gas Production, Ruminal Fermentation, Methane Production and Ruminal Digestibility Based on an *in vitro* Technique

Manlin Wei, Liping Ren, Zhenming Zhou and Qingxiang Meng
State Key Laboratory of Animal Nutrition, College of Animal Science and Technology,
China Agricultural University, 100193 Beijing, P.R. China

Abstract: A study was conducted to investigate the effect of addition of three plant extracts on *in vitro* gas production, ruminal fermentation, methane production and ruminal digestibility. Three plant extracts including Tea Saponin (TS), Mulberry Leaf Extract (MLE) and Ecdysterone (ECD) were added to the substrate at 1.6, 1.25 and 0.2 mg g^{-1}, respectively. When plant extracts were added all the plant extracts showed a similar pattern of Gas Production (GP). Compared with Control (CON), MLE and ECD had numerically higher GP at all time points of incubation but TS showed higher GP only at 1st 28 h incubation and then decreased afterwards. Shorter lag time and faster rate of gas production were observed for TS than ECD, MLE and CON, although the difference was not significant ($p>0.05$). No matter what time points were taken, each of plant extracts did not exhibit significant changes in methane production and proportion ($p>0.10$). Compared with CON, the addition of plant extracts did not change *in vitro* ruminal pH , ammonia concentrations, total VFA ($p>0.05$) and the ratio of acetate to propionate ($p>0.10$) but decreased molar proportions of both butyrate and minor acid (including valerate, isobutyrate and isovalerate) ($p<0.05$), Digestibilities of DM (IDDM) and OM (IDOM) were 38.0 and 35.3%, 41.3 and 44.9%, 39.1 and 38.0% and 41.3 and 37.2% for CON, TS, MLE and ECD, respectively. Although, plant extracts of TS, MLE and ECD did not significantly ($p>0.05$) change the IDDM relative to CON, TS had higher IDOM than CON ($p<0.05$). It is concluded that tea saponin showed a potential value as a plant extract feed additive used by ruminant animals. Further study is needed to provide more information about animal performance responses to the addition of plant extracts to the diets of ruminants.

Key words: Tea saponin, mulberry leaf extract, ecdysterone, gas production, ruminal fermentation, methane production, ruminal digestibility

INTRODUCTION

The attempts of using plant extracts in animal feeds and animal health agents have been carried out for many years because of the worldwide use of plant extracts as traditional medicines for human beings, especially with the legislation (1831/2003; EC, 2003) introduced within the European Union to prohibit the use of growth-promoting antibiotics in animal feeds. Because plant extracts were believed to be natural, safe and efficient without the hormonal consequences or negative side effects, more interests were turned to alternative plant extracts instead of growth-promoters antibiotics. Consequently, several plant extracts acquired more attentions due to their superior effects.

Tea Saponin (TS) is a vast group of secondary compounds produced from tea plants with active substances of triterpenoid and steroids. Besides its surfactant property, TS has a haemolytic properties for its interaction with the sterols of the erythrocyte membrane and sterol-binding capacity on protozoa membranes which causes the destruction of protozoa (Hart *et al*., 2008). TS was considered to have an effect on rumen fermentation, especially on a diet (or substrate) rich in grain or starch (Hristov *et al*., 1999; Lila *et al*., 2003). Mulberry Leaf Extract (MLE) derived active principals from mulberry (*Morus alba*) leaves which were used as the feeds in sericulture in China for centuries is also an important traditional Chinese herbal medicine. Mulberry leaves contain a variety of active ingredients including alkaloids, polysaccharide, flavonoid glycoside, steroids, etc. and was proved to have the inhibitory effect of diabetic, hypertension, oxidants and weight loss. Ecdysterone (ECD) a natural steroid hormone from plants mostly abundant with the 20 hydroxyecdysone and its analogues has been recognized for its positive pharmacological properties in mammals of antihyperglycemia, antihyperlipidemia, hepatoprotective

Corresponding Author: Qingxiang Meng, State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, 100193 Beijing, P.R. China

action, antioxyditive, etc. (Slama and Lafont, 1995; Dinan and Lafont, 2006) as well as an anabolic effect of promoting protein synthesis (Dinan, 2001; Bizec *et al*., 2002). Moreover, it was reviewed that ECD has an antimicrobial activity on bacteria and fungi (Lafont and Dinan, 2003).

Commercial products of these plant extracts are easy to access for their extensive sources and economic price in China, suggesting their potential application in practice. The use of these plant extracts in livestock feeding, however was still in the initial stage and the knowledge of their effect on livestock, especially on rumen fermentation characteristics and nutrient digestibility was rather scarce. Therefore, this study was conducted to investigate the effect of the addition of plant extracts on gas production, ruminal fermentation, methane production and ruminal digestibility based on an *in vitro* technique.

MATERIALS AND METHODS

Plant extracts: TS, MLE and ECD were commercially purchased from Hangzhou Tangtian Technology Co., Ltd. Xi'an Sai'ao Biotechnology Co., Ltd. and Kunming Shancao Bioengineering Co., Ltd. of China, respectively. The contents of effective components are 30, 40 and 10%, respectively. TS, MLE or ECD were added to the incubation substrate at 1.6, 1.25 and 0.2 mg g^{-1}, respectively.

Inoculum and substrate: Inoculum was prepared according to the method of Menke and Steingass (1988). Briefely, rumen fluid was obtained from three Limousin steers (average body weight about 450 kg) installed with a permanent rumen cannula before morning feeding and strained through four layers of cheesecloth. Inoculums were the mixture of rumen fluid with buffer at the ratio of 1:2. All procedures involving animals were conducted under the approval of the China Agricultural University Animal Science and Technology College Animal Care and Use Committee. The ration of the cattle consisted of 35% corn stalk silage, 20% brewer's grains, 40.8% ground corn, 1.5% red dates meal, 0.6% soybean meal, 0.7% limestone meal, 0.1% dicalcium phosphate, 0.6% sodium bicarbonate, 0.5% salt and 0.2% mineral premix. The substrate used in the *in vitro* fermentation was the same as the ration of experimental steers but air dried and finely ground to pass through a 1 mm screen.

***In vitro* gas production and methane production:** *In vitro* Gas Production (GP) was determined according to the method of Menke and Steingass (1988). Briefely, the substrate of approximately 200 mg Dry Matter (DM), plant extract and 30 mL inoculums were added to the 100 mL calibrated glass-syringe (Model HFT000025, Haberle Labortechnik, Germany) and incubated in a water bath shaker at 39°C. GP (mL) was recorded at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44 and 48 h incubation, respectively.

Triplicate syringes of each treatment were taken out of the incubator at 12, 24 and 48 h incubation and placed into cold water with ice cubes to terminate the fermentation. Gas was sampled by 5 mL airtight syringe and methane proportion (%, v/v) was measured by gas chromatography (TP-2060T, Beijing Analytical Instrument Co., Ltd. China) equipped with a TCD detector (column: TDX-01, 1 m×3 mm×2 mm, column temperature: 120°C, detector temperature: 150°C, injector temperature: 150°C, carrier gas: argon; gas flow: 50 mL min^{-1}, injection volume: 0.1 mL). And the methane production (mL) was calculated through corresponding GP.

Ruminal fermentation parameters: Inoculum in the syringes was removed into 50 mL centrifuge tubes at 24 h incubation after gas sampling and the pH values were immediately determined (teso205, Testo AG, Germany). The inoculum was centrifuged (8000×g for 15 min at 4°C) and the supernatant was analyzed for ammonia nitrogen concentration (Wang *et al*., 2011a, b) and the volatile fatty acids (Liu *et al*., 2001) using gas chromatography (GC 3420, Beijing Analytical Instrument Factory, China) equipped with a FID detector (column: PEG-20M+H_3PO_4, 2 m×6 mm×2 mm, column temperature: 145°C, detector temperature: 200°C, injector temperature: 200°C, carrier gas: Nitrogen; gas flow: 30 mL min^{-1}, injection volume: 0.6 μL).

***In vitro* ruminal digestibility of dry matter and organic matter of the diet:** The *In vitro* ruminal Digestibility of Dry Matter (IDDM) and Organic Matter (IDOM) of the diet was investigated at 24 h incubation according to the first step of the method of Tilley and Terry (1963).

Statistical analysis: Gas production data were fitted to the model of Orskov and McDonald (1979).

$$Y = B \times \left(1 - e^{-c(t-lag)}\right)$$

Where:

Y = The GP (mL)
B = Predicted maximum GP (mL)
c = Rate of gas production (mL h^{-1})
t = Incubation time (h)
lag = lag phase (h)

Analysis of variance and multiple comparisons of the data were conducted using the General Linear Models (GLM) procedure and Studen Newman Keuls (SNK) test of SAS 8.1.

RESULTS AND DISCUSSION

Effect of plant extract on *in vitro* gas production: Gas production volumes (mL/200 mg DM) in different incubation time are shown in Fig. 1. When plant extracts were added to the substrates, all the extracts showed a similar pattern of gas production. Compared with Control (CON), MLE and ECD had numerically higher GP at all time points of incubation but TS showed higher GP only at 1st 28 h incubation and then decreased afterwards.

The data of gas production parameters (B, c, lag) are shown in Table 1. The predicted maximum GP of plant extracts are accordant with the fashion of actual GP shown in Fig. 1. Compared with CON, MLE tended to have increased potential gas production ($p = 0.09$) whereas there were no differences in maximum GP between CON, TS and ECD ($p>0.1$). Shorter lag time and faster rate of gas production were observed for TS than for ECD, MLE and CON suggesting that TS can improve rumen fermentation of certain ingredients in the substrate although the difference was not significant ($p>0.05$).

Effect of plant extract on methane production: The data of methane production and proportion (%, v/v) are shown in Table 2. No matter what time points were concerned, each of plant extracts did not exhibit significant changes in methane production and their proportion ($p>0.10$) although, the methane proportion for all plant extracts slightly lower than the control at each of time points.

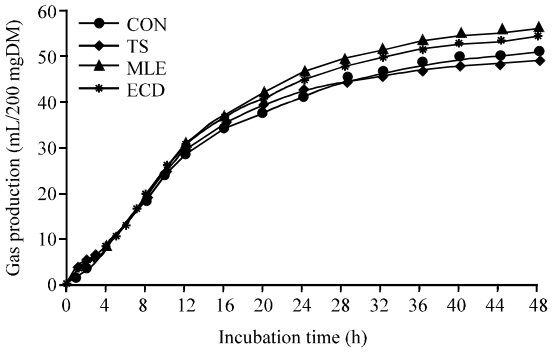

Fig. 1: *In vitro* gas production in 48 h incubation; CON: Control; TS: Tea Saponion; MLE: Mulberry Leaf Extract; ECD: Ecdysterone

Table 1: The gas production parameters as affected by plant extract addition

	Treatment					
Items	CON	TS	ECD	MLE	SEM	p-value
B (mL/200 mg DM)	54.800	53.700	58.700	61.500	3.400	0.394
c (mL h^{-1})	0.059	0.069	0.059	0.056	0.008	0.747
L (h)	0.760	0.510	0.710	0.740	0.100	0.295

B: maximum GP; c: Rate of gas production; L: Lag phase; CON: Control; TS: Tea Saponin; MLE: Mulberry Leaf Extract; ECD: Ecdysterone

Effect of plant extract on *in vitro* ruminal fermentation parameters: The results of *in vitro* ruminal fermentation parameters at 24 h incubation are shown in Table 3. Compared with CON, the addition of all the plant extracts did not change *in vitro* ruminal pH, ammonia-nitrogen concentrations and total VFA ($p>0.05$) but significantly alter some of individual VFA compositions ($p<0.05$). When plant extracts were added, there were decreases in molar proportions of both butyrate and minor acids (including valerate, isobutyrate and isovalerate) ($p<0.05$) but there were no changes ($p>0.10$) in the proportions of acetate and propionate and consequently the ratio of acetate to propionate.

Effect of plant extract on *in vitro* ruminal digestibility: Digestibilities of DM (IDDM) and OM (IDOM) were 38.0, 35.3, 41.3, 44.9, 39.1, 38.0, 41.3 and 37.2% for CON, TS, MLE and ECD, respectively as shown in Fig. 2. Although, plant extracts of TS, MLE and ECD did not significantly ($p>0.05$) change the IDDM relative to CON, TS had higher IDOM than CON ($p<0.05$) whereas MLE and ECD did not differ significantly compared with CON or TS ($p>0.10$).

A great number of plant extracts were investigated as rumen fermentation regulators to improve utilization of

Table 2: Methane proportion and production as affected by plant extract addition in the incubation of 12, 24 and 48 h

	Treatment					
Items	CON	TS	MLE	ECD	SEM	p-value
Methane production (mL/200 mg DM)						
12 h	5.8	4.6	5.5	6.0	0.5	0.206
24 h	8.3	8.1	8.7	7.7	0.5	0.630
48 h	15.6	13.6	14.0	14.0	1.0	0.545
Methane proportion (% v/v)						
12 h	20.4	15.6	18.1	19.8	1.6	0.196
24 h	20.2	19.2	18.8	17.2	1.3	0.461
48 h	30.9	27.9	25.1	25.8	2.1	0.267

CON: Control; TS: Tea Saponin; MLE: Mulberry Leaf Extract; ECD: Ecdysterone

Table 3: Ruminal fermentation parameters as affected by plant extract addition at 24 h incubation

	Treatment					
Items	CON	TS	MLE	ECD	SEM	p-value
pH	6.45	6.49	6.52	6.51	0.02	0.175
NH_3-N (mg/100 mL)	27.02	26.09	24.45	20.77	2.30	0.301
TVFA (mmol L^{-1})	51.95^{ab}	56.07^{a}	49.63^{ab}	47.08^{b}	1.68	0.028
VFA molar proportion (%)						
Acetate	65.34	66.72	65.33	66.17	0.50	0.220
Propionate	17.63	18.20	18.78	18.72	0.36	0.163
Butyrate	11.89^{a}	10.55^{b}	11.26^{ab}	10.60^{b}	0.22	0.008
Minor acid	5.14^{a}	4.54^{b}	4.63^{b}	4.51^{b}	0.13	0.031
A:P	3.71	3.67	3.48	3.54	0.10	0.336

NH_3-N: Ammonia Nitrogen; VFA: Volatile Fatty Acid; TVFA: Total Volatile Fatty Acid; Minor acid including valerate, isobutyrate and isovalerate; A:P, Acetate to Propionate ratio. CON: Control; TS: Tea Saponin; MLE: Mulberry Leaf Extract; ECD: Ecdysterone. Values in the same row with different letters mean significant difference ($p<0.05$)

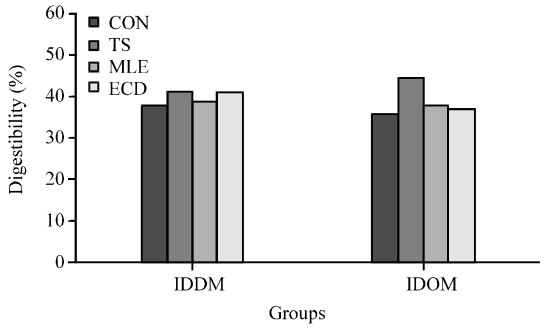

Fig. 2: *In vitro* ruminal diagestibilities of dry matter and organic matter at 24 h incubation. IDDM: *In vitro* Digestibility of Dry Matter; IDOM: *In vitro* Digestibility of Organic Matter; CON: Control; TS: Tea Saponion; MLE: Mulberry Leaf Extract; ECD: Ecdysterone

feedstuff in the past few decades. In the current study, tea saponin, mulberry leaf extract and ecdysterone were studied for their effects on rumen fermentation *in vitro*.

The trend of decreasing *in vitro* gas production by the addition of TS observed in this study is consistent with the result of earlier studies that TS or saponin-containing substances have an effect of reducing total gas production and methane production (Lila *et al*., 2003; Hu *et al*., 2005; Wang *et al*., 2011a, b). The *in vitro* gas production by the addition of both MLE and ECD tended to be increased in the beginning of the incubation although, it was not significant ($p>0.05$). Researchers observed the increased potential gas production by the addition of mulberry leaves (Table 1) which is similar to the result of Liu *et al*. (2001). However, the reports about the effect of MLE and ECD on rumen fermentation were rather absent except some researchers reported the pharmacological and biological functions of plants contain these two plant extracts (Slama and Lafont, 1995; Liu *et al*., 2001). Regardless of decreased or increased *in vitro* gas production owing to the addition of plant extracts, the methane production was reduced in some extent which indicated inhibitory effects on methane production by plant extracts. The reason for these suppressive effects on methane production by plant extracts may be related to its direct and indirect inhibition of growth of protozoa, methanogens and fungi in the rumen. The similar results were reported in other studies using TS as a plant extract additive (Hristov *et al*., 1999; Guo *et al*., 2008; Klita *et al*., 1996; Hess *et al*., 2003a, b; Kamra *et al*., 2006). The differences in the suppressive magnitude between the current study and other experiments are presumed due to not only the level of the extract addition but also the composition of the substrates. Whether MLE and ECD had the same effect as TS on ruminal microbial growth would not be validated at present although studies using mulberry leaves and plant contain ECD exhibited some suppressive effects on certain microbes (Slama and Lafont, 1995; Ratanapo *et al*., 2001). In fact, the pellet mixture of mulberry leaf powder, urea and some binding agent was found to have an effect on rumen microorganism population (Tan *et al*., 2012). Similarly, the ecdysone (another kind of ecdysteriod) was also found to improve the productivity of ruminants by elimination of rumen protozoal growth from digestive tract (Slama and Lafont, 1995). Since, methane production in the rumen results in the loss of energy of diet and contributes to global greenhouse gas emissions, the suppression of methane emission from the rumen by plant extracts would be meaningful in promoting animal performance and improving the environment.

The ruminal fermentation parameters *in vitro* were changed by the addition of plant extracts (Table 3). The molar proportion of butyrate and other minor acids were significantly decreased by the addition of plant extracts and the molar proportion of propionate tended to increase although it was not significant. The similar results were observed in other studies using TS as a feed additive in ruminants (Guo *et al*., 2008; Mao *et al*., 2010; Wang *et al*., 2009). While some studies showed that addition of plant extracts would result in an alteration of predominant microbes in the rumen, especially the absence of protozoa (Eugene *et al*., 2004; Morgavi *et al*., 2008) and consequently in changes of their interaction with other species and the hydrogen transfer pathway (Kamra *et al*., 2006) the result was not observed in this study based on the unchanged methane production (Table 2) and the unchanged ratio of acetate to propionate (Table 3). In some studies, researchers reported that NH_3-N concentration was decreased (Wang *et al*., 2009; Zhou *et al*., 2011) and ruminal pH was increased (Hu *et al*., 2005) by TS administration. However, the results seemed to be not the case in this study. On the other hand, the trends of decreased methane proportion, TVFA, butyrate, minor acid and NH_3-N as well as the increased propionate proportion and pH values in the incubation of 24 h for MLE and ECD than those for CON are very similar to the effect of defaunation (Eugene *et al*., 2004) which suggests that there could be an inhibitory effect of MLE and ECD on protozoa to certain extent just as the defaunation agents.

The IDOM was increased by TS addition which was accordant with the shorter lag time and faster rate of gas production (Table 1) and the numerically higher TVFA (Table 3). This result indicated that TS treatment could improve the rumen fermentation of organic matter. It was

presumed due to the growing number of bacteria associated with the suppressive effect of protozoa. Simultaneously, although, it was not significant, the IDDM and IDOM was numerically increased by MLE and ECD additions which was consistent with the increase in the gas production due to their additions (Fig. 1), suggesting a higher utilization of components in substrates.

CONCLUSION

The addition of plant extracts results in a decreased molar proportion of butyrate and minor acids but does not change methane production and ruminal fermentation pattern. Compared with control, mulberry leaf extract and ecdysterone, tea saponin would increase ruminal VFA production, gas production rate and *in vitro* OM digestibilities. Overall, tea saponin appears to have a potential value as a plant extract feed additive used by ruminant animals. Further study is needed to provide more information about animal performance responses to the addition of plant extracts to the diets of ruminants.

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