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Effects of mulberry leaf flavonoid and resveratrol on methane emission and nutrient digestion in sheep

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ABSTRACT

As a new type of methane control agent, natural plant extract has been widely studied in recent years, but *in vivo* studies are few. This study was to investigate the effects of the dietary supplementation of 2 different polyphenols on the methane (CH_4) emission and digestion metabolism in sheep. Ten healthy crossbred sheep (Dorper ♂ × small-tailed Han ♀; BW 60.0 ± 1.73 kg) were used in a change-over design. The sheep were fed the following 3 diets in the present study: the basal diet (CON) with no supplementation; the basal diet supplemented with 2 g mulberry leaf flavonoid (MLF) per day per sheep; the basal diet supplemented with 0.25 g resveratrol (RES) per day per sheep. Both MLF and RES reduced CH_4 emission scaled to metabolic weight per kilogram of DMI and CO_2 output scaled to metabolic weight, but the effect of RES was significant ($P < 0.05$). Both MLF and RES significantly improved apparent digestibility of DM, OM, NDF, ADF, and nitrogen, but the effect of RES was significant ($P < 0.05$). Both MLF and RES significantly improved ME ($P < 0.05$) and reduced energy losses in CH_4 emission ($P > 0.05$). In conclusion, MLF and RES can improve the digestibility of nutrients, the utilization of nutrients and energy, and reduce CH_4 emission, but they are not conducive to nitrogen retention.

1. Introduction

Due to global warming and climate change, greenhouse gas emission has been a considerable concern. As one of main greenhouse gases, methane (CH_4) accounted for 15 to 20% contributions to global warming (Wang and Wen, 1996; You and Liao, 2004). Methane has been known to be the second most anthropogenic greenhouse gas, which is after carbon dioxide (CO_2), but has 21 times global warming potential of CO_2 (UNFCCC, 2006). Agriculture was responsible for about 47% of total anthropogenic emissions of CH_4, and CH_4 from enteric fermentation in livestock accounted for 32% of the total (IPCC, 2007). A total of 7.7×10^7 t CH_4 emission in agricultural production each year was from ruminants and rumen fermentation, which accounted for 90% of the total (IPCC, 1996). Approximately 95.5% of the CH_4 produced by feed fermentation in the rumen was exhaled through noses and mouths of ruminants (AGO, 2003). Methane emission was also one of the main ways that energy was lost during fermentation. Depending on different diets, the amount of energy loss by CH_4 emission represented a 2 to 12% energy loss of feed (Johnson and Johnson, 1995). Therefore, to reduce CH_4 emissions by ruminants is of great significance not only in mitigating climate warming but also in efficient use of feed in livestock production. As a new type of CH_4 control agent, natural plant extract has been widely studied in recent years. Adding 200 mg Yucca extract per kilogram diet reduced urea nitrogen and significantly increased the metabolic rate of dietary protein (Tang, 2004). Adding *Lespedeza*, which was rich in tannins, to the Spanish wether diets directly affected the activity of methanogens, thereby reduced CH_4 production (Animut et al., 2008). Adding *Ligustrum lucidum* extract to diets increased DM, OM, CP, NDF, and ADF digestibility and reduced the urea nitrogen excretion, thus improved the efficiency of protein (Xu, 2007). The effects of catechin and resveratrol (RES) on rumen fermentation had been

studied *in vitro*, and the results showed that they could reduce CH_4 production (Becker and van Wikselaar, 2011). Many *in vitro* studies have indicated that natural plant extract has a role in terms of inhibiting CH_4. However, there are few *in vivo* studies. Thus, we used adult sheep, fed them plant extracts, which were extracted from mulberry leaf flavonoid (MLF) and RES, and measured CH_4 and CO_2 emissions using an open-circuit respiratory system. Based on the fact that MLF and RES have influences on CH_4 emission, we conducted a further research on the metabolism of the sheep.

2. Materials and methods

This study was conducted from April to June 2013 at the Experimental Station of the Chinese Academy of Agricultural Sciences (CAAS), Beijing, China. The sample analysis was conducted in the laboratory of nutrition and physiology of domestic animals in CAAS in August 2013.

2.1. Chemicals and equipment

Natural plant extracts: MLF, extracted from mulberry leaves, the purity was 5% using ultraviolet (UV); RES, extracted from knot weed, the purity was 98% using high performance liquid chromatography (HPLC). They were obtained by the following procedures.

Mulberry leaf flavonoid: high quality, clean and dry mulberry leaves were extracted using 85°C, 80% CH_3CH_2OH → refluxed for 1.5 to 2 h to obtain ethanol extract → extracted twice under the same condition → collected extracts → concentrated → dried → 80 mesh crushed → sample testing → packed.

Resveratrol: high quality, clean, dry and crushed roots of Polygonum cuspidatum were extracted → standing → filtered → concentrated to obtain a polydatin crude extract → extracted with ethyl acetate → HPLC → obtained high purity crude RES → repeatedly recrystallized with ethanol to obtain a white crystalline solid RES (purity > 98%) → dried → crushed → sample testing → packed.

The gas metabolism test device (Sable Systems International, Las Vegas, NV, USA) consisted of an open-circuit respirometry system, a closed gas metabolism chamber, and matching calculation software.

2.2. Animal management

Ten primiparous ewes (Dorper × small-tailed Han, BW 60.0 ± 1.73 kg) were used. Following diets were offered to the sheep in 3 experiments: 1) the basal diet with no supplementation (CON); 2) the basal diet supplemented with 2 g MLF per day per sheep; 3) the basal diet supplemented with 0.25 RES per day per sheep. The basal diet included pelleted total mixed rations (TMR) and Chinese wild-rye hay (Table 1). The sheep were fed 1,500 g TMR at 0800 h, and 200 g of Chinese wild-rye hay at 1200 h daily. The feeding level fulfilled the maintenance and growth requirements of yearling sheep (BW 60 kg) according to the NRC (2007). All animals were housed in individual pens and had free access to fresh water during whole experimental period.

2.3. Experimental procedures

The sheep were transferred to metabolism crates for a 7-d adaptation period. The excreta and urine of each ewe was collected for 8 d. The amount of feed, orts, and feces was weighed and homogenized daily. After that, 10% of it was sampled and stored at −20°C until analysis. The urine of each sheep was also collected to a bucket containing 100 mL 10% (vol/vol) H_2SO_4 daily. After volume measurements, 10 mL/L of the total was sampled and stored

Table 1
Ingredients and nutrient composition of the basal diet (air-dry basis).

Item	Content
Ingredients, %	
Chinese wild-rye hay	68.66
Corn	17.00
Soybean meal	12.00
$CaHPO_4$	1.35
Limestone	0.25
NaCl	0.50
Premix[1]	0.24
Nutrient composition, %[2]	
DM	88.60
CP	12.25
Ether extract	2.71
Crude ash	6.32
Gross energy, MJ/kg	17.20
Metabolizable energy, MJ/kg	8.77
NDF	41.36
ADF	21.78
Calcium	0.87
Phosphorus	0.39

[1] The premix provided the following amount per kilogram of diets: Cu 15.0 mg, Fe 100.0 mg, Mn 60.0 mg, Zn 100.0 mg, I 0.9 mg, Se 0.3 mg, Co 0.2 mg; VA 16,000 IU, VD 4,000 IU, VE 100 IU.
[2] The nutrition values were measured.

at −20°C until analysis. All samples of the 8 d for each ewe were mixed to form a composite for analysis.

Methane production was determined using the open-circuit respirometry system with 3 metabolism cages. Each cage was fitted with a polycarbonate head box. On d 0, 2, 4, and 6 in the 8-d collection period, the sheep were transferred to the metabolism cages that equipped with head boxes for the CH_4 output assessments. After a 24-h adaptation period, CH_4 production from each sheep was measured in turn in 24 h as described by Deng et al. (2012).

2.4. Sample analysis

The DM content was measured after the samples were dried in an air-forced oven at 135°C for 2 h. The ash content was measured after the samples were dried in a muffle furnace at 550°C for 24 h. The OM content, as the difference between DM and ash contents, was measured. The protein nitrogen content of feedstuffs is generally 16%, thus we deduced the protein content according to the measured nitrogen content and the fixed nitrogen-to-protein (N:P) conversion factor is 6.25. Nitrogen was measured according to the methods of Kjeldahl, thus we can calculated crud protein. The GE was measured using a bomb calorimeter (C200, IKA Works Inc., Staufen, Germany). The NDF and ADF were measured according to Van Soest et al. (1991) and Goering and Van Soest (1970), respectively.

Generally, we use the apparent digestibility of nutrients to represent the digestion and absorption of nutrients. According to Feng (2004), nutrients apparent digestibility = (total nutrients intake − nutrients in faces)/total nutrients intake, DE = gross energy intake (GEI) − fecal energy losses (FE), and ME = GEI − FE − urinary energy losses (UE) − energy losses in CH_4 emission (CH_4E), where the CH_4E is mainly from rumen fermentation and the heat of CH_4 is 890.3 kJ/mol or 39.75 kJ/L.

2.5. Statistical analyses

Data were analyzed using one-way ANOVA by SAS (SAS Institute Inc, 2005). Significant differences were accepted when $P < 0.05$.

3. Results

3.1. The effect of MLF and RES on CH_4 emission

Table 2 shows that RES supplementation decreased daily CO_2 emission (L/d), CO_2 emission scaled to metabolic weight (L/kg $W^{0.75}$), daily CH_4 emission (L/d), CH_4 emission per kilogram of DMI (L/kg DMI), CH_4 emission scaled to metabolic weight (L/kg $W^{0.75}$), and CH_4 emission scaled to metabolism weight per kilogram of DMI (L/kg $W^{0.75}$ kg DMI) compared with CON ($P < 0.05$). However, MLF supplementation had no effect on the above parameters ($P > 0.05$).

3.2. The effect of MLF and RES on digestion and metabolism

3.2.1. Apparent digestibility of DM and OM

Table 3 shows that RES supplementation decreased DM in feces ($P < 0.05$), increased digested DM and DM apparent digestibility ($P < 0.05$), and increased digested OM and OM apparent digestibility ($P < 0.05$). However, MLF supplementation had no effect on the apparent digestibility of DM and OM ($P > 0.05$).

3.2.2. The apparent digestibility of NDF and ADF

Table 4 shows that RES supplementation significantly increased the digested NDF and ADF ($P < 0.05$), and significantly increased the apparent digestibility of NDF and ADF ($P < 0.05$). In contrast, MLF supplementation had no effect in these aspects ($P > 0.05$).

3.2.3. Nitrogen metabolism

Table 5 shows that RES supplementation significantly decreased faecal nitrogen, significantly increased N digestibility and significantly increased urinary nitrogen output ($P < 0.05$), thus no difference in N retention was observed ($P > 0.05$). However, MLF supplementation had no apparent effect on N metabolism.

3.2.4. Energy metabolism

Table 6 shows both MLF and RES supplementation decreased GEI scaled to metabolic weight ($P > 0.05$), significantly decreased fecal energy losses and FE scaled to metabolic weight ($P < 0.05$), and decreased energy loses in CH_4E ($P > 0.05$). Mulberry leaf supplementation decreased urinary energy losses scaled to metabolic weight and CH_4E:GEI ratio ($P > 0.05$), in contrast, RES supplementation significantly decreased in these aspects ($P < 0.05$). Both MLF and RES significantly increased DE, ME, DE:GEI ratio and ME:GEI ratio ($P < 0.05$). Mulberry leaf supplementation increased DE scaled to metabolic weight, ME scaled to metabolic weight, and ME:DE ratio ($P > 0.05$), in contrast, RES supplementation significantly increased in these aspects ($P < 0.05$).

4. Discussion

4.1. Effects on CH_4 emission

Methane emission from ruminants relates to their unique digestive characteristics. There are a large number of cellulolytic bacteria, CH_4 bacteria and other anaerobic microorganisms existing in the rumen. Dietary carbohydrates and other plant fibers, after being swallowed and going through anaerobic fermentation in the rumen, are broken down into volatile fatty acids, hydrogen and CO_2, etc. These chemicals, including CO_2, formic, acetic, methylamine and dimethylamine, participate in the production of CH_4 under the influence of methanogens. Methane that was produced in the gastrointestinal tract of ruminants could hardly be digested by animals, thus it was excreted through breathing or belching (Hao et al., 2000).

Studies on the effect of plant extracts on CH_4 production by ruminants were mostly conducted in vitro. Effects of Yucca saponin on CH_4 production in vitro was reported by Wang and McAllister (1998), and the results showed that it reduced CH_4 emissions by 15% compared with the control group. Busquet et al. (2005) reported that adding garlic oil (300 mg/L rumen fluid) reduced methanogenesis by 74%, and adding diallyl disulfide (300 mg/L rumen fluid) reduced methanogenesis by 69%. In the present study, CH_4 emission was measured directly in feeding trials and the results showed that RES reduced CH_4 emissions by 10.64%. These results showed the difference of plant extracts supplementation between in vitro and in vivo studies.

Resveratrol belongs to non-flavonoid polyphenolic compounds and its chemical structure contains a plurality of phenyl and hydroxyl. It is a natural and active constituent in plants such as Polygonum cuspidatum. The potential mechanisms by which plant extracts reduce CH_4 generation include the impact of fiber degradation (Waghorn et al., 2002), the promotion of propionic acid production (Calsamiglia et al., 2007), and the inhibition of ciliates

Table 2
Gas metabolism and methane (CH_4) emission in sheep fed different diets

Item	Diets[1]		
	CON ($n = 10$)	MLF ($n = 5$)	RES ($n = 5$)
DMI, g	1,512.45 ± 0.06	1,512.36 ± 0.06	1,512.51 ± 0.04
Metabolic weight, kg $W^{0.75}$	21.56 ± 0.15[b]	22.65 ± 0.52[a]	23.47 ± 0.19[a]
DMI/$W^{0.75}$, g/kg $W^{0.75}$	70.19 ± 0.48[a]	67.15 ± 1.80[ab]	64.49 ± 0.52[b]
O_2 consumption			
O_2, L/d	543.46 ± 6.39	571.19 ± 18.08	537.53 ± 24.91
O_2, L/kg $W^{0.75}$	25.22 ± 0.35[a]	25.23 ± 0.54[a]	22.91 ± 1.05[b]
CO_2 emission			
CO_2, L/d	541.75 ± 6.95	535.72 ± 17.91	504.39 ± 28.29
CO_2, L/kg $W^{0.75}$	25.15 ± 0.41[a]	23.69 ± 0.69[ab]	21.49 ± 1.18[b]
Respiratory quotient	1.00 ± 0.01	0.94 ± 0.02	0.94 ± 0.03
CH_4 emission			
CH_4, L/d	61.15 ± 0.64	60.48 ± 2.76	59.57 ± 2.43
CH_4, L/kg DMI	40.43 ± 0.43	39.99 ± 1.82	39.45 ± 1.61
CH_4, L/kg $W^{0.75}$	2.84 ± 0.04[a]	2.66 ± 0.08[ab]	2.54 ± 0.09[b]
CH_4/$W^{0.75}$, L/(kg $W^{0.75}$·kg DMI)	1.88 ± 0.03[a]	1.76 ± 0.05[ab]	1.68 ± 0.06[b]

kg $W^{0.75}$ = body weight (kg) raised to the power 0.75.
[a, b] Within a row, means with different superscripts differ significantly ($P < 0.05$).
[1] CON = the basal diet; MLF = the basal diet supplemented with 2 g mulberry leaf flavonoids per day per animal; RES = the basal diet supplemented with 0.25 g resveratrol per day per animal.

Table 3
The apparent digestibility of DM and OM in sheep fed different diets.

Item	Diets[1]		
	CON ($n = 10$)	MLF ($n = 5$)	RES ($n = 5$)
DM			
DMI, g/d	1,512.54 ± 0.06	1,512.51 ± 0.04	1,512.62 ± 0.04
DM in faeces, g/d	591.83 ± 20.28[a]	608.04 ± 3.04[a]	529.05 ± 28.68[b]
Digested DM, g/d	920.70 ± 20.29[b]	904.46 ± 3.01[b]	983.57 ± 28.67[a]
Apparent digestibility,%	60.87 ± 1.34[b]	59.80 ± 0.20[b]	65.02 ± 1.90[a]
OM			
OM intake, g/d	1,549.82 ± 0.06	1,549.79 ± 0.05	1,549.91 ± 0.04
OM in faeces, g/d	616.04 ± 8.52[a]	607.86 ± 4.78[a]	488.00 ± 26.89[b]
Digested OM, g/d	933.79 ± 8.52[b]	941.93 ± 4.75[b]	1,061.91 ± 26.88[a]
Apparent digestibility, %	60.25 ± 0.55[b]	60.78 ± 0.31[b]	68.51 ± 1.73[a]

[a, b] Within a row, means with different superscripts differ significantly ($P < 0.05$).
[1] CON = the basal diet; MLF = the basal diet supplemented with 2 g mulberry leaf flavonoids per day per animal; RES = the basal diet supplemented with 0.25 g resveratrol per day per animal.

Table 4
The apparent digestibility of NDF and ADF in sheep fed different diets.

Item	Diets[1]		
	CON ($n = 10$)	MLF ($n = 5$)	RES ($n = 5$)
NDF			
NDF intake, g/d	762.17 ± 0.04	762.15 ± 0.03	762.22 ± 0.03
NDF in faeces, g/d	473.49 ± 8.53[a]	490.77 ± 17.47[a]	339.22 ± 17.18[b]
Digested NDF, g/d	288.69 ± 8.54[b]	271.38 ± 17.47[b]	423.00 ± 17.18[a]
Apparent digestibility, %	37.88 ± 1.12[b]	35.61 ± 2.29[b]	55.50 ± 2.25[a]
ADF			
ADF intake, g/d	403.12 ± 0.02	403.11 ± 0.02	403.15 ± 0.01
ADF in faeces, g/d	246.78 ± 5.03[a]	260.51 ± 11.72[a]	183.13 ± 9.10[b]
Digested ADF, g/d	156.35 ± 5.05[b]	142.60 ± 11.71[b]	220.02 ± 9.10[a]
Apparent digestibility,%	38.78 ± 1.54[b]	35.38 ± 2.91[b]	54.58 ± 2.26[a]

NDF = neutral detergent fiber; ADF = acid detergent fiber.
[a, b] Within a row, means with different superscripts differ significantly ($P < 0.05$).
[1] CON = the basal diet; MLF = the basal diet supplemented with 2 g mulberry leaf flavonoids per day per animal; RES = the basal diet supplemented with 0.25 g resveratrol per day per animal.

(Hu et al., 2005). In this study, RES reduced CH_4E by probably increasing the passing rate of chyme and nutrients through the rumen. In the rumen fermentation process, with the generation of acetic acid, a large amount of H_2 and CO_2 was produced. Methanogens in turn used the hydrogen and CO_2 to produce CH_4. When the ratio of acetate to propionic acid decreased, the utilization of hydrogen was enhanced, thus the CH_4 production was decreased. In addition, a facultative relationship existed between Methanogenic bacteria and ciliates. Thereby RES likely inhibited the growth of ciliates and reduced CH_4E. *Flavonoids Staphylococcus* inhibited aureus, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* (Fukui et al., 1988). Sophora isoprene flavanone derivatives has a significant antibacterial effect on gram-positive bacteria (Gao and Wang, 2005). Phenolic compounds with hydroxyl groups and enzyme active sites forms hydrogen bonds which are highly antibacterial (Franz et al., 2010; Calsamiglia et al., 2007; Burt et al., 2004). Therefore, RES could directly reduce or inhibit the activity of methanogens, thereby reduce CH_4 production. Becker and van Wikselaar (2011) used *in vitro* test and showed that adding RES reduced CH_4 production.

4.2. Effects on digestion and metabolism

Many plant extracts were reported to improve digestion and absorption of nutrients by altering animal gut microflora (Hernandez et al., 2004). Chen et al. (1999) described that daidzein improved ruminal digestibility. Addition of mulberry leaves also reduced the feed conversion ratio and increased protein concentration in Mutton sheep (Li, 2012). Supplementation of tea flavonoids enhanced weight gain and reduced the incidence of respiratory disease in Dorset sheep, reported by Zhang et al. (2005). The beneficial effect of mulberry leaves on lactation was also proved (Benavides, 2000). In human body, RES was extensively metabolized in the small intestine, modified to glucuronides or other metabolites in the liver, and finally excreted in urine (Walle et al., 2004; Requena et al., 2010).

Table 5
Nitrogen metabolism in sheep fed different diets

Item	Diets[1]		
	CON ($n = 10$)	MLF ($n = 5$)	RES ($n = 5$)
Metabolic weight, kg $W^{0.75}$	21.56 ± 0.15[b]	22.65 ± 0.52[a]	23.47 ± 0.19[a]
NI, g/d	32.13 ± 0.00	32.13 ± 0.00	32.13 ± 0.0
NI/$W^{0.75}$, g/(kg $w^{0.75}$ · d)	1.50 ± 0.01[a]	1.39 ± 0.01[b]	1.37 ± 0.01[b]
FN, g/d	10.72 ± 0.14[a]	10.03 ± 0.14[a]	9.08 ± 0.51[b]
FN/$W^{0.75}$, g/(kg $w^{0.75}$ · d)	0.50 ± 0.01[a]	0.43 ± 0.01[b]	0.39 ± 0.02[c]
UN, g/d	14.87 ± 0.37[a]	16.13 ± 0.33[a]	17.11 ± 0.48[b]
UN/$W^{0.75}$, g/(kg $w^{0.75}$ · d)	0.69 ± 0.02	0.70 ± 0.02	0.73 ± 0.02
Nitrogen apparent digestibility, %	66.63 ± 0.42[b]	68.78 ± 0.43[b]	71.74 ± 1.59[a]
NR, g/d	6.54 ± 0.37	5.97 ± 0.34	5.95 ± 0.41
NR/$W^{0.75}$, g/(kg $w^{0.75}$ · d)	0.30 ± 0.02	0.26 ± 0.01	0.25 ± 0.02
NR:NI ratio	20.34 ± 1.17	18.58 ± 1.05	18.50 ± 1.28

kg $W^{0.75}$ = body weight (kg) raised to the power 0.75; NI = nitrogen intake; FN = faecal nitrogen; UN = urinary nitrogen; NR = nitrogen retention.
[a, b] Within a row, means with different superscripts differ significantly ($P < 0.05$).
[1] CON = the basal diet; MLF = the basal diet supplemented with 2 g mulberry leaf flavonoids per day per animal; RES = the basal diet supplemented with 0.25 g resveratrol per day per animal.

Table 6
Performances on the energy apparent digestibility and metabolism in sheep fed different diets.

Item	Diet[1]		
	CON ($n = 10$)	MLF ($n = 5$)	RES ($n = 5$)
Metabolic weight, kg $W^{0.75}$	21.56 ± 0.15^b	22.65 ± 0.52^a	23.47 ± 0.19^a
GEI, MJ/d	29.33 ± 0.00	29.33 ± 0.00	29.33 ± 0.00
GEI/$W^{0.75}$, MJ/(kg $W^{0.75} \cdot$ d)	1.37 ± 0.01	1.27 ± 0.01	1.25 ± 0.01^b
FE, MJ/d	12.58 ± 0.16^a	11.12 ± 0.05^b	9.79 ± 0.53^c
FE/$W^{0.75}$, MJ/(kg $W^{0.75} \cdot$ d)	0.59 ± 0.01^a	0.48 ± 0.01^b	0.42 ± 0.02^c
UE, MJ/d	0.78 ± 0.05	0.80 ± 0.05	0.66 ± 0.07
UE/$W^{0.75}$, MJ/(kg $W^{0.75} \cdot$ d)	0.04 ± 0.00^a	0.03 ± 0.00^{ab}	0.03 ± 0.00^b
CH$_4$E, MJ/d	2.44 ± 0.02	2.41 ± 0.07	2.36 ± 0.10
CH$_4$E/$W^{0.75}$, MJ/(kg $W^{0.75} \cdot$ d)	0.11 ± 0.00^a	0.11 ± 0.00^{ab}	0.10 ± 0.00^b
DE, MJ/d	16.75 ± 0.16^c	18.21 ± 0.05^b	19.55 ± 0.53^a
DE/$W^{0.75}$, MJ/(kg $W^{0.75} \cdot$ d)	0.78 ± 0.01^b	0.79 ± 0.01^b	0.83 ± 0.03^a
ME, MJ/d	13.53 ± 0.16^c	14.93 ± 0.07^b	16.53 ± 0.54^a
ME/$W^{0.75}$, MJ/(kg $W^{0.75} \cdot$ d)	0.63 ± 0.01^b	0.65 ± 0.01^b	0.71 ± 0.03^a
DE:GEI ratio	57.10 ± 0.54^c	62.08 ± 0.18^b	66.62 ± 1.79^a
ME:GEI ratio	46.13 ± 0.53^c	50.90 ± 0.24^b	56.36 ± 1.82^a
ME:DE ratio	80.77 ± 0.34^c	81.99 ± 0.50^b	84.47 ± 0.70^a
CH$_4$E:GEI ratio	8.31 ± 0.06	8.21 ± 0.24	8.04 ± 0.33

kg $W^{0.75}$ = body weight (kg) raised to the power 0.75; GEI = gross energy intake; FE = fecal energy losses; UE = urinary energy losses; CH$_4$E = energy loses in CH$_4$ emission.

[a, b] Within a row, means with different superscripts differ significantly ($P < 0.05$).

[1] CON = the basal diet; MLF = the basal diet supplemented with 2 g mulberry leaf flavonoids per day per animal; RES = the basal diet supplemented with 0.25 g resveratrol per day per animal.

Studies showed that flavonoids improved feed intake, growth performance, the absorption and utilization of nutrients, immune function, and the development of mammary gland, as well as lactation (Arjmandi et al., 2000; Weaver and Zafar, 2004; Zhang et al., 2006). The phenolic hydroxyl in the structure of polyphenol compounds and its number and position could affect the affinity of related enzymes and transporters, which in turn affected the absorption and metabolism of nutrients in intestine (Teng, 2007). The present study showed that RES supplementation improved the apparent digestibility of DM, OM, NDF and ADF. The following are 3 possible reasons: 1) The increase of saliva secretion lead to the increase of bioactive peptides, thereby improved the digestion and absorption of nutrients. Polyphenols are substrates for several enzymes, including hydrolyzing and conjugating enzymes, and they are located in the small intestine and colon (Landete, 2012). Therefore, RES might also improve nutrient digestibility by participating in the synthesis of certain enzymes in the small intestine of sheep. 2) Changes in rumen fermentation. There is a wide variety of microorganisms in the rumen, which could change the gastrointestinal flora and in turn improve the digestion and metabolism of nutrients. 3) Effect on metabolism mechanism. Studies showed that diets containing ipriflavone isoflavones affected the neuroendocrine system of castrated piglets by increasing testosterone, insulin-like growth factor-1 (IGF-1) and serum calcium levels (Han et al., 2006). Studies also showed that, RES was mostly metabolized to glucuronide in the gastrointestinal tract of human and absorbed into the blood where it exerted its physiological action (Henry et al., 2005).

4.3. Effects on energy metabolism

To our knowledge, there are no published studies related to the use of MLF or RES in ruminants. This study used the open-circuit respiratory system to measure CH$_4$E. The calculated CH$_4$E accounted for 8.04 to 8.11% of the gross energy of diet. The results showed that supplementation of plant extracts reduced faecal energy and CH$_4$E, accordingly improved the apparent digestible energy,

apparent metabolizable energy, DE:GE ratio, ME:GE ratio, and ME:DE ratio. These parameters represented the energy utilization efficiency of the sheep. An animal's need for energy is mainly supplied by nutrient oxidization. Usually this is realized by the three cycles of system implementation, namely glycolysis cycle, citric acid cycle, oxidative phosphorylation (Feng, 2004). Plant extracts that were studied in this experiment have their own special chemical structure, which may play important roles in the energy metabolism pathway, in terms of following reasons: 1) the ketone type carbonyl of MLF and the benzene ring and the hydroxyl group of RES provided electrons to the three major electronic circulation, which promoted the oxidation–reduction reaction. 2) Through a redox reaction, a keto carbonyl group or a benzene ring and a hydroxyl group formed a quinone reaction system (two-electron reaction system), which promoted the occurrence of the entire electron transport system, and contributed to energy metabolism pathway. 3) Acetyl coenzyme A (CoA) was an intermediate product of the three major cycling routes, and MLF or RES could promote the rumen VFA generation, which therefore acted on the CoA and promoted energy metabolism. 4) The reduction in the number of methanogenic bacteria. Methane was produced by methanogenic bacteria using CO$_2$ and H$_2$ which were produced by the rumen carbohydrate fermentation. As the number of methanogenic bacteria was reduced, CH$_4$E was decreased, then energy loss was decreased, thereby the diet utilization efficiency was improved.

5. Conclusions

Under the experimental conditions, RES decreased CH$_4$E by 10.64%, decreased N deposition rate, increased nutrient digestibility, and improved the apparent digestibility of energy. However, the effect of MLF on above parameters in the present study was not obvious.

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