Effects of chestnut tannins and coconut oil on growth performance, methane emission, ruminal fermentation, and microbial populations in sheep

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ABSTRACT

This study was conducted to evaluate the effects of chestnut tannins (CT) and coconut oil (CO) on growth performance, methane (CH₄) emission, ruminal fermentation, and microbial populations in sheep. A total of 48 Rideau Arcott sheep (average body weight 31.5 ± 1.97 kg, 16 wk old) were randomly assigned into 6 treatment groups in a 3 × 2 factorial design, with CT and CO as the main effects (8 sheep per group). The treatments were control diet (CTR), 10 or 30 g of CT/kg of diet (CT10 and CT30), 25 g of CO/kg of concentrate (CO25), and 10 or 30 g of CT/kg of diet + 25 g of CO/kg of concentrate (CT10CO25 and CT30CO25). After the feeding trial (60 d), all sheep were moved to respiratory chambers to measure CH₄ emission. After CH₄ emission measurements, all sheep were slaughtered to obtain rumen fluid samples. Results showed that the addition of CT, CO, and CT + CO had no significant effects on growth performance of sheep but reduced CH₄ emission. Addition of CT reduced the NH₃-N concentration in rumen fluid in CT30. Addition of CO decreased the concentration of total volatile fatty acids in rumen fluid. No significant differences were observed in pH and molar proportion of volatile fatty acids among treatments. Addition of CT, CO, and CT + CO significantly decreased methanogen and protozoa populations. Moreover, CO decreased counts of *Fibrobacter succinogenes*. No significant differences were observed in populations of fungi, *Ruminococcus flavefaciens*, or *Ruminococcus albus* among treatments. In conclusion, supplementation of CT and CO seemed to be a feasible means of decreasing emissions of CH₄ from sheep by reduction of methanogen and protozoa populations with no negative effect on growth performance.

Key words: chestnut tannin, coconut oil, sheep, rumen ecology

INTRODUCTION

Increasing greenhouse gas emissions continues to be a great concern due to their potential adverse effects on global warming and climate change (IPCC, 2007). Methane (CH₄) is one of the greenhouse gases and it has 23 times more global warming potential than carbon dioxide (IPCC, 2007). Methane is usually produced in the process of anaerobic enteric fermentation of feeds in animals, especially ruminants. It is estimated that about 95% of global animal enteric CH₄ is from ruminants (Johnson and Ward, 1996). Moreover, 2 to 15% of ingested gross energy is converted to CH₄ during ruminal fermentation in ruminants, which reduces the efficiency of feedstuff utilization (Johnson and Johnson, 1995). Therefore, mitigation of CH₄ emissions is receiving a great deal of attention, especially through dietary strategies. Several studies have indicated that dietary manipulation could reduce CH₄ production in the rumen by inhibiting rumen microbes involved in CH₄ formation or by diverting hydrogen away from CH₄ formation (McGinn et al., 2004; Mao et al., 2010).

Tannins are a complex group of water-soluble polyphenolic compounds, which are classified, in general, into 2 groups: hydrolysable tannins and condensed tannins. Tannins have been reported to modulate fermentation patterns, reduce CH₄ production, and reduce rumen protozoa counts (Patra et al., 2006; Grainger et al., 2009). Bhatta et al. (2009) evaluated in vitro effects of chestnut tannins (CT; extracted from chestnut wood, *Castanea sativa*, which is rich in hydrolysable tannins) on CH₄ production and found that CT reduced CH₄ production by 5.5% and suppressed the population of methanogenic archaea by 12.0%. However, little is known about the in vivo effects of CT on CH₄ production and ruminal microorganisms. Moreover, decreasing CH₄ formation could lead to the accumulation of hydrogen, and unsaturated fatty acids (UFA) might provide an alternative hydrogen sink (Czerkawski et al., 1966). Previous studies have reported that some vegetable oils rich in UFA decreased CH₄ production and altered fermentation patterns due to biohydrogenation and toxic effects of medium-chain or polyunsaturated fatty acids

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on methanogens (Prins et al., 1972; McGinn et al., 2004) and protozoa (Broudiscou et al., 1990; Mao et al., 2010). Coconut oil (CO) contains approximately 6.2% monounsaturated fatty acids and 1.6% polyunsaturated fatty acids, so it seems to be an effective hydrogen acceptor and inhibitor of microorganisms involved in methanogenesis. However, no information on a combined analysis of tannins and CO has been reported. Thus, we hypothesized that the effects of CT and CO on inhibiting methane emissions would be additive and designed this study to investigate the effects of dietary additions of CT and CO on growth performance, CH₄ emissions, ruminal fermentation, and microbial populations in sheep.

MATERIALS AND METHODS

Animals, Diets, and Experimental Designs

All procedures were approved by the Administration Office of Laboratory Animals, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences. A total of 48 Rideau Arcott sheep (average BW of 31.5 ± 1.97 kg, 16 wk old) were randomly divided into 6 treatment groups in a 3×2 factorial design with CT and CO as main effects (8 sheep per group). The treatments were control diet (CTR), 10 g of CT/kg of diet (CT10), 30 g of CT/kg of diet (CT30), 25 g of CO/kg of concentrate (CO25), 10 g of CT/kg of diet + 25 g of CO/kg of concentrate (CT10CO25), and CT 30 g of CT/kg of diet + 25 g of CO/kg of concentrate (CT30CO25). The control diet was formulated according to the recommended values from NRC (2007; Table 1). Dry matter was determined by oven drying at 105°C overnight (AOAC, 1995; method 930.15). Ether extract (EE) was determined using a Soxhlet apparatus (AOAC, 1995; method 945.16). Crude protein was measured by a Kjeldahl nitrogen analysis (AOAC, 1995; method 954.01). Contents of NDF and ADF were determined as explained by Van Soest et al. (1991) using heat-stable amylase (A3306, Sigma, St. Louis, MO) and sodium sulfite, and expressed without residual ash. Gross energy (GE) content was determined using the values for heat of combustion by an adiabatic bomb calorimeter (Parr Instrument Co., 1970). To prepare the experimental diet, appropriate amounts of CO were added over several minutes while mixing silage, and then concentrate (or mixed with chestnut tannins) was added and mixed with the oil-containing silage. The EE contents were 2.4, 2.4, 2.3, 3.5, 3.4, and 3.2% on a DM basis in the CTR, CT10, CT30, CO25, CT10CO25, and CT30CO25 diets, respectively. The CT (SilvaTeam, San Michele di Mondovì, Italy) was extracted from chestnut wood by a heat and low-pressure treatment

according to the manufacturer's information; only the water-soluble fraction was retained and subsequently dehydrated. The product was presented as a fine brown powder. Chemical composition of the batch used in this experiment was as follows: 75.4% tannins (14% condensed tannins), 20.7% nontannin, 2.5% water, and 1.4% insolubles (pH 3.26, 0.1 mg/mL solution) on a fresh matter basis. The total tannin contents, expressed as tannic acid equivalents, were measured according to the Folin-Ciocalteu method (Makkar et al., 1993). The condensed tannins content was determined as described by Porter et al. (1986). The fatty acid profile of CO was measured using GC as described by Mir et al. (1999) (Table 1).

From d 1 to 60 of the trial, 48 sheep were housed individually in 48 uniform pens equipped with a feeder and a water bowl. Sheep had free access to feed and water. Feed intake and BW were individually recorded weekly from d 1 to 60 of the trial to calculate the ADG, DMI, and feed conversion ratio (FCR).

After the feeding trial, 48 sheep were moved to 48 respiratory chambers to measure CH₄ emission (one sheep per chamber). Measurements lasted 4 d. After

Table 1. Ingredients and chemical composition of the control diet and the fatty acid (FA) profile of coconut oil

Item	Amount
Ingredient, % of DM	
Barley silage	52
Rolled barley	38
Soybean meal	8
Dicalcium phosphate	0.4
Limestone	0.7
Trace mineralized salt ¹	0.87
Vitamin mix ²	0.03
Chemical composition, % of DM	
DM	62.1
CP	15.2
Ether extract	2.4
NDF	33.2
ADF	21.1
Gross energy, MJ/kg of DM	17.9
Fatty acids profile of coconut oil, % of total FA	
C6:0	1.22
C8:0	8.34
C10:0	5.76
C12:0	42.2
C14:0	17.7
C16:0	8.43
C18:0	3.75
C18:1	9.56
C18:2	2.62
C18:3	0.12
C20:0	0.10
C20:1	0.17

¹Contained 93.01% NaCl, 5% mineral mix (20% S, 18% K, 11% Mg, 1 g of Fe/kg), 0.92% ZnSO₄·4H₂O, 0.13% CuSO₄·5H₂O, 0.014% ethylenediamine dihydroiodide, 0.003% Na₂SeO₃, and 0.005% CoSO₄·6H₂O.

²Contained 10,000 IU vitamin A, 1,000 IU vitamin D, 100 IU vitamin E.

1 d adaptation period, CH₄ emissions were measured continuously for 72 h, as described in the following section. Sheep was tethered in the central location. Feed intake and BW were individually recorded at the end of the 72 h measurement. After measurement of CH₄ emission, all sheep were slaughtered to obtain rumen samples for further analyses.

Respiratory Chamber and Methane Measurement

Respiratory chambers (3.0 m wide × 3.5 m length × 2.5 m tall, 26.25 m³ volume) were vented using a fresh air intake and an exhaust duct, with a blower at the inlet. The inlet was set on the upper side of one wall and the outlet on the bottom of the opposite wall. The volume of airflow through the chamber was recorded by using a flow meter (ST50, Fluid Components International, San Marcos, CA) set at the inlet. The air volume within the chamber was exchanged every 10 min. During the 72 h of measurements, 50-mL air samples from the inlet and outlet of each chamber were taken once every 2 h using glass gas syringes (Poulten & Graf Ltd., Barking, UK). After sampling, gas samples were transferred to Tedlar gas bags (Haide Technologies Ltd., Dalian, China) and sent to the laboratory for immediate analysis. The concentration of CH₄ in the collected gas samples was analyzed in a gas chromatograph (GC-8A, Shimadzu Corp., Kyoto, Japan) with a stainless steel column (80/100 mesh Porapak Q column) and a C-R3A integrator (Kajikawa et al., 2007). Gas samples were dried using a Nafion dryer. Carrier gas was nitrogen (N₂) at 29 kPa. The column ran isothermally at 45°C. Calibration was performed using a standard gas mixture (50.2 ppm CH₄ in N₂, Messer Griesheim, Dusseldorf, Germany).

Methane emissions were calculated according to the equation $CH_4 = (C_2 - C_1) \times Q \times 60 \times 24 \times 16 / 22.4$, where C₁ and C₂ are the CH₄ concentrations (L/L) of gas sample from inlet and outlet, respectively; Q is ventilation rate (L/min); 60 is minutes in an hour and 24 is hours in a day; 16 is the molecular weight of CH₄ (g/mol); and 22.4 is the volume of 1 mol of methane (L/mol). The average CH₄ emission from each chamber was calculated as the average of the emissions in 36 sampling points. Before measurements, methane recovery from the chambers was measured to calibrate the chamber. We released the standard methane gas (99.99%, 2.625 m³/min) into 12 chambers, and the measured methane recoveries of these chambers exceeded 0.88. Temperature and relative humidity within the chamber were determined using the AcuRite Thermometer with Humidity Gauge (Chaney Instrument Co., Lake Geneva, WI). The average temperature within the chamber was 25°C and the relative humidity ranged from 40 to 75%.

Ruminal Fermentation Measurement

Rumen fluid was collected immediately after slaughtering, and then strained through 4 layers of compress gauze. The pH of rumen liquor was determined using a pH meter (Crison Instruments, Barcelona, Spain). Volatile fatty acids were determined by GC (GC-8A, Shimadzu Corp.) as described by Hu et al. (2005). Ammonia-N was determined colorimetrically with a Technicon Auto Analyzer (Shimadzu Corp.; Hu et al., 2005).

Total DNA Extraction, Primers, and Real-Time Quantitative PCR

Six aliquots of 1 mL of rumen fluid were sampled and stored in liquid nitrogen immediately for determination of the relative quantity to total bacterial 16S rDNA of methanogens, protozoa, fungi, *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Fibrobacter succinogenes*. Total DNA was extracted from rumen fluid by physical disruption using a bead-beating method as described by Zhang et al. (2008). In brief, the method involved resuspending the microbial cells in a lysis buffer in tubes containing zirconium beads. Tubes were bead-beaten at $3,901 \times g$ for 3 min in a mini-bead beater (Biospec Products, Bartlesville, OK). After centrifugation of the sample at $14,000 \times g$ for 15 min at 4°C, the supernatant was mixed with the supplied glass milk solution and washed before a final elution step, which released the DNA from the glass milk. All amplifying primer sets described as Denman and McSweeney (2006) and Denman et al. (2007) are presented in Table 2. The real-time quantitative PCR was performed using the ABI 7900 real-time PCR system (Applied Biosystems, Foster City, CA), with fluorescence detection of SYBR Green dye. Amplification consisted of 1 cycle of 50°C for 2 min and 95°C for 2 min for initial denaturalization, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Detection of the fluorescent product was set at the last step of each cycle. To determine the specificity of amplification, analysis of product melting was conducted after each amplification. The melting curve was obtained by slow heating with a 0.1°C/s increment from 60°C to 95°C, with fluorescence collection at 0.1°C intervals. Amplification efficiencies for each primer pairs were investigated by examining dilution series of total rumen microbial DNA template on the same plate in triplicate (methanogens 100.32%, protozoa 95.73%, fungi 96.83%, *R. albus* 93.23%, *R. flavefaciens* 89.37%, and *F. succinogenes* 90.34%). The populations of methanogens, protozoa, fungi, *R. albus*, *R. flavefaciens*, and *F. succinogenes* were expressed as a proportion of total rumen bacterial 16S rDNA.

Table 2. Primers for real-time quantitative PCR assay

Target species	Primer sequence ¹
Methanogens	Forward: TTCGGTGGATCDCARAGRGC Reverse: GBARGTCGWAWCCGTAGAATCC
Fungi	Forward: GAGGAAGTAAAAGTCGTAACAAGGTTTC Reverse: CAAATTCACAAAGGGTAGGATGAATT
Protozoa	Forward: GCTTTTCGWTGGTAGTGTATT Reverse: CTTGCCCTCYAATCGTWCT
<i>Ruminococcus albus</i>	Forward: CCCTAAAAGCAGTCTTAGTTCG Reverse: CCTCCTTGCGGTTAGAACA
<i>Ruminococcus flavefaciens</i>	Forward: CGAACGGAGATAATTTGAGTTTACTTAGG Reverse: CGGTCTCTGTATGTTATGAGGTATTACC
<i>Fibrobacter succinogenes</i>	Forward: GTTCGGAATTACTGGGCGTAAA Reverse: CGCCTGCCCCTGAACATC

¹D = A/G/T; R = A/G; B = C/G/T; W = A/T; Y = C/T.

Statistical Analysis

All data were statistically analyzed by ANOVA using the MIXED model procedure of SAS (version 9.1.3, SAS Institute Inc., Cary, NC). For all variables, the model tested for the fixed effect of CT and CO, and the interaction of CT and CO. For CH₄ emission data, day was a random variable and chamber was treated as a repeated term within diet. Means were compared by Tukey's multiple range tests when the main effects were significant. Statistical significance among means was accepted at $P < 0.05$.

RESULTS AND DISCUSSION

Growth Performance and Methane Emission

No significant CT effects were observed on ADG, DMI, and FCR (Table 3). Our findings were similar to work conducted by Al-Dobaib (2009), who found that a diet treated with quebracho tannins at the level of 1 or 3% of DM did not affect ADG, DMI, and FCR of lambs. Likewise, Obeidat et al. (2011) observed that replacement of part of barley grain by tannin-rich carob pods did not affect ADG, DMI, and FCR of Awassi lambs. In addition, no significant CO and CT + CO effects were observed on ADG, DMI, and FCR (Table 3). Similar results were observed by Bhatt et al. (2011), who found that CO supplementation (25, 50, and 75 g/kg of concentrate) had no effects on ADG, DMI, or FCR of lambs during the postweaning period. Conversely, Machmüller and Kreuzer (1999) observed that the addition of 70 g/kg of dietary CO reduced DMI of lambs. They suggested that one possible reason for this finding could be reduced palatability. Because of the depression in feed intake, Sutton et al. (1983) suggested that the maximum CO in sheep diet should be limited to 40 g/d per animal.

In the present study, CT affected the emission of CH₄ from sheep (Table 3). Average emissions of CH₄ from sheep fed the CT30 diet (45.85 g/animal per day or 23.58 g/kg of DMI/d) were significantly lower than that from sheep fed the CTR or CT10 diet (61.18 g/animal per day or 31.07 g/kg of DMI/d and 57.10 g/animal per day or 29.19 g/kg of DMI/d, respectively; $P < 0.05$). Previously, the inhibitory effect of CT on CH₄ production was reported by Bhatta et al. (2009), who observed that CT reduced in vitro CH₄ production by 19.5%. Waghorn et al. (2002) reported that incubation of *Lotus pedunculatus* containing tannins had lower CH₄ production (in vitro) compared with *L. pedunculatus* plus a tannin binder. Moreover, sheep fed *L. pedunculatus* produced 17% less CH₄ compared with sheep fed *L. pedunculatus* plus a tannin binder. The effect of tannin on suppressing methanogenesis was mostly attributed to decreasing the methanogenic population in rumen fluid (Bhatta et al., 2009).

In the current study, average CH₄ emissions from sheep fed diets with CO supplementation were lower than those from sheep fed diets without CO supplementation ($P < 0.05$, 51.87 g/animal per day or 26.51 g/kg of DMI/d vs. 57.55 g/animal per day or 29.38 g/kg of DMI/d). This result was consistent with Machmüller and Kreuzer (1999), who found that supplementation of CO at proportions of 3.5 and 7% on a DM basis suppressed CH₄ production from sheep by 28 and 73%, respectively, compared with the unsupplemented diet. Likewise, Kongmun et al. (2011) suggested that supplementation of 7% CO on a DM basis reduced CH₄ production in swamp buffalo by 10%. Previous researchers reported that UFA had toxic effects on methanogens and protozoa involved in CH₄ formation (Matsumoto et al., 1991; Mao et al., 2010). This toxic effect of CO might be responsible for CH₄ suppression (Machmüller et al., 2003). Moreover, a significant CT + CO effect was

Table 3. Growth performance and CH₄ emission of sheep (n = 8) fed diets with chestnut tannins (CT), coconut oil (CO), or diets with chestnut tannins plus coconut oil

Treatment ¹	Initial BW (kg)	Final BW (kg)	ADG (g/d)	DMI (g/d)	Feed conversion ratio (g/g)	DMI in chamber (g/d)	Methane	
							Average emission (g/animal per day)	Average emission (g/kg of DMI/d)
CTR	29.5	47.4	298	1,483	4.97	1,966	65.8 ^a	33.4 ^a
CT10	29.1	47.3	303	1,466	4.84	1,953	58.4 ^{ab}	29.8 ^a
CT30	30.2	47.9	295	1,453	4.93	1,948	48.5 ^{bc}	24.9 ^{bc}
CO25	30.0	48.5	308	1,503	4.98	1,970	56.5 ^b	28.7 ^a
CT10CO25	29.9	48.6	311	1,451	4.66	1,955	55.8 ^b	28.6 ^{ab}
CT30CO25	29.6	47.6	300	1,431	4.77	1,940	43.3 ^c	22.3 ^c
SEM	0.59	1.63	13.2	46.2	0.211	48.3	1.82	0.61
Main effect								
CT								
CT0	29.8	47.9	303	1,493	4.98	1,968	61.2 ^a	31.1 ^a
CT10	29.5	48.0	307	1,459	4.75	1,954	57.1 ^a	29.2 ^a
CT30	29.9	47.8	298	1,442	4.85	1,944	45.9 ^b	23.6 ^b
CO								
CO0	29.6	47.5	299	1,467	4.91	1,956	57.6 ^a	29.4 ^a
CO25	29.9	48.2	306	1,462	4.81	1,955	51.9 ^b	26.5 ^b
P-value								
CT	0.84	0.63	0.73	0.10	0.46	0.24	0.02	0.04
CO	0.93	0.87	0.73	0.89	0.18	0.99	0.03	0.05
CT + CO	0.89	0.67	0.58	0.64	0.49	0.75	0.01	0.03

^{a-c}Means in the same section with different superscripts differ ($P < 0.05$).

¹CTR = control diets; CT10 = 10 g of CT/kg of diet; CT30 = 30 g of CT/kg of diet; CO25 = 25 g of CO/kg of concentrate; CT10CO25 = 10 g of CT/kg of diet + 25 g of CO/kg of concentrate; CT30CO25 = 30 g of CT/kg of diet + 25 g of CO/kg of concentrate.

observed on average CH₄ emission from sheep expressed as gram/animal per day ($P < 0.01$) or gram/kilogram of DMI/day ($P < 0.05$). We can explain this positive associative effect in 2 ways: first, the UFA included in CO are good electron acceptors for hydrogen diverted from the CH₄ suppressing effects of CT (Czerkawski et al., 1966), and second, CT and UFA have toxic effects on methanogens and protozoa involved in CH₄ formation (Bhatta et al., 2009; Mao et al., 2010).

Rumen Fermentation Characteristics

No differences were found in pH in rumen fluid among treatments (Table 4), which fell within the normal range for sheep (Firkins, 1996). These results were in agreement with the observation of Bhatta et al. (2009), who reported that addition of CT in feed did not affect the pH of rumen fluid in vitro. Similar results were observed in an in vivo experiment (Krueger et al., 2010). Moreover, Kongmun et al. (2011) observed that addition of CO did not affect the pH in rumen fluid of sheep. Conversely, Machmüller et al. (2003) observed a significant decrease in the pH in rumen fluid of sheep fed a diet with CO supplementation.

In the present study, sheep fed the CT30 diet had lower rumen fluid NH₃-N concentrations ($P < 0.05$) compared with sheep fed the CTR or CT10 diet. However, no CO and CO + CT effects were observed

on NH₃-N concentration among treatments (Table 4). Similar results were reported by Bhatta et al. (2007) in sheep, by Bhatta et al. (2005) in goats, and by Min et al. (1998) in ewes. Three possible mechanisms explain these findings. First, CT might reduce the protein degradability in the rumen because it could form complexes with protein (Tabacco et al., 2006). Second, the activity of microbial deaminase might be inhibited by tannins (Leinmuller and Menke, 1990). Third, the bacteria-degrading activity of protozoa might be inhibited by tannins (Jouany, 1994).

In the current study, addition of CO decreased the total VFA concentration in rumen fluid ($P < 0.05$), whereas no CT or CT + CO effects were observed among treatments (Table 4). Moreover, no differences were observed in the molar proportion of VFA among treatments (Table 4). Previous studies on tannins showed that feeding tannins did not affect total VFA concentration and molar proportion of VFA in ewes (Hervás et al., 2003), wether lambs (Nunez-Hernandez et al., 1991), and goats (Zimmer and Cordesse, 1996). In contrast, Bhatta et al. (2009) found that tannins (5% of TMR on a DM basis) extracted from mimosa tended to reduce total VFA production and increase the molar proportion of propionate in vitro. Concerning the effect of CO on total VFA production, the results of this study were consistent with those of Machmüller et al. (2003) and Kongmun et al. (2011). Moreover, CO

Table 4. pH, ammonia nitrogen (NH₃-N), VFA concentrations, and molar proportions in rumen fluid of sheep fed diets with chestnut tannins (CT), coconut oil (CO), or diets with chestnut tannins plus coconut oil (n = 8)

Treatment ¹	pH	NH ₃ -N (mg/L)	Total VFA (mmol/L)	Molar proportion (mol/100 mol)						
				Acetate (A)	Propionate (P)	Isobutyrate	Butyrate	Isovalerate	Valerate	A:P
CTR	6.86	174.6 ^a	107.4 ^a	64.3	19.7	1.03	11.23	1.53	2.21	3.26
CT10	6.79	167.7 ^a	104.2 ^a	64.0	19.6	1.10	11.32	1.67	2.31	3.27
CT30	6.81	128.5 ^b	108.5 ^a	63.9	19.9	1.09	11.01	1.62	2.48	3.21
CO25	6.85	173.3 ^a	92.3 ^b	64.1	20.1	1.00	10.89	1.58	2.33	3.19
CT10CO25	6.90	163.7 ^a	90.3 ^b	63.6	20.4	1.11	11.02	1.62	2.26	3.12
CT30CO25	6.87	123.6 ^b	91.2 ^b	63.5	20.4	1.08	11.12	1.60	2.30	3.11
SEM	0.04	10.23	3.72	0.39	0.31	0.083	0.102	0.089	0.090	0.078
Main effect										
CT										
CT 0	6.85	173.9 ^a	99.8	64.2	19.9	1.02	11.06	1.56	2.27	3.23
CT 10	6.85	165.7 ^a	97.3	63.8	20.0	1.11	11.17	1.65	2.29	3.20
CT 30	6.84	131.1 ^b	99.9	63.7	20.2	1.09	11.07	1.61	2.39	3.16
CO										
CO0	6.82	156.9	106.7 ^a	64.1	19.7	1.07	11.19	1.61	2.33	3.25
CO25	6.87	153.5	91.3 ^b	63.7	20.3	1.06	11.01	1.60	2.30	3.14
P-value										
CT	0.90	0.04	0.82	0.39	0.33	0.49	0.88	0.32	0.47	0.90
CO	0.27	0.82	0.04	0.46	0.10	0.92	0.80	0.95	0.82	0.75
CT + CO	0.76	0.47	0.15	0.25	0.13	0.75	0.44	0.88	0.65	0.33

^{a-c}Means in the same section within a parameter with different superscripts differ ($P < 0.05$).

¹CTR = control diets; CT10 = 10 g of CT/kg of diet; CT30 = 30 g of CT/kg of diet; CO25 = 25 g of CO/kg of concentrate; CT10CO25 = 10 g of CT/kg of diet + 25 g of CO/kg of concentrate; CT30CO25 = 30 g of CT/kg of diet + 25 g of CO/kg of concentrate.

showed effects on acetate and propionate proportion (Kongmun et al., 2011), which was different from our results.

Rumen Microbes

Effects on rumen microbial population are shown in Table 5. The populations of methanogens, protozoa, fungi, and *F. succinogenes* in the CTR group were similar to other reports in sheep (methanogens: 0.24–0.61% of total bacterial 16S rDNA, protozoa: 4.68–9.71% of total bacterial 16S rDNA, fungi: 0.02–0.05% of total bacterial 16S rDNA, *F. succinogenes*: 0.68–1.29% of total bacterial 16S rDNA; Mao et al., 2010; Zhou et al., 2011). However, the populations of *R. flavefaciens* and *R. albus* in the CTR group exceeded values of other reports (*R. flavefaciens*: 0.03–0.15% of total bacterial 16S rDNA, *R. albus*: 0.03–0.05% of total bacterial 16S rDNA, Mao et al., 2010; Zhou et al., 2011).

In the present study, addition of CT reduced methanogen ($P < 0.05$) and protozoa ($P < 0.05$) populations. No CT effects were observed on fungi, *R. albus*, *R. flavefaciens*, or *F. succinogenes* populations. Previously, Tavendale et al. (2005) demonstrated an inhibitory effect of tannins extracted from *L. pedunculatus* on the growth of a pure culture of rumen methanogens. Animut et al. (2008) also observed lower methanogen populations in the rumen of goats fed diets containing tannins. On the contrary, Bhatta et al. (2009) observed

that inclusion of a mimosa tannin sample containing 7.78% hydrolysable tannins and 1.5% condensed tannins at 250 g/kg of mixed feed substrates did not reduce rumen methanogen populations. A possible reason for the antimethanogenic effect is the inhibitory effect of tannins on the activities of enzymes necessary for the growth of methanogens (Patra and Saxena, 2011). Moreover, similar to our results, several studies (Wang et al., 1994; Patra et al., 2006; Bhatta et al., 2009) found that tannins had antiprotozoal properties. It is well known that methanogens are associated symbiotically with the ciliate protozoa on the surface and inside the protozoa (Finlay et al., 1994). Thus, a reduction in protozoa populations might also cause a reduction in methanogen populations.

Addition of CO reduced methanogen ($P < 0.05$) and protozoa ($P < 0.05$) populations. The CO + CT interaction was also observed on the reduction of methanogen ($P < 0.05$) and protozoa ($P < 0.05$) populations. Similar results were obtained by Kongmun et al. (2011), who fed swamp buffalo with 7% CO supplementation and found decreased total methanogen and protozoa populations in rumen fluid. Likewise, Dohme et al. (1999) reported that the number of methanogen and protozoa per milliliter of rumen fluid was reduced by using CO instead of protected fat. The reduction of methanogen and protozoa populations may be due to the toxicity of UFA on methanogen and protozoa (Prins et al., 1972; Zhang et al., 2008).

Table 5. Microorganism population in rumen fluid of sheep (n = 8) fed diets with chestnut tannins (CT), coconut oil (CO), or diets with chestnut tannins plus coconut oil (% total bacterial 16S rDNA)

Treatment ¹	Methanogens	Protozoa	Fungi ($\times 10^{-2}$)	<i>Ruminococcus albus</i> ($\times 10^{-1}$)	<i>Ruminococcus flavefaciens</i>	<i>Fibrobacter succinogenes</i>
CTR	0.37 ^a	10.11 ^a	5.72	1.82	0.31	1.31 ^a
CT10	0.27 ^{bc}	6.21 ^{ab}	4.52	1.93	0.33	1.28 ^a
CT30	0.24 ^{bc}	5.14 ^b	4.12	1.84	0.34	1.30 ^a
CO25	0.28 ^b	5.04 ^b	4.18	1.78	0.29	0.79 ^b
CT10CO25	0.26 ^{bc}	5.00 ^b	3.89	1.87	0.33	0.82 ^b
CT30CO25	0.22 ^c	4.72 ^b	3.66	1.90	0.41	0.84 ^b
SEM	0.021	0.982	0.961	0.141	0.158	0.091
Main effect						
CT						
CT0	0.33 ^a	7.58 ^a	4.95	1.80	0.30	1.05
CT10	0.27 ^b	5.61 ^{ab}	4.21	1.90	0.33	1.05
CT 30	0.23 ^b	4.93 ^b	3.89	1.87	0.38	1.07
CO						
CO0	0.29 ^a	7.15 ^a	4.79	1.86	0.33	1.30 ^a
CO25	0.25 ^b	4.92 ^b	3.91	1.85	0.34	0.81 ^b
P-value						
CT	0.05	0.03	0.08	0.65	0.89	0.47
CO	0.04	0.02	0.16	0.93	0.96	0.04
CT + CO	0.04	0.04	0.09	0.76	0.65	0.69

^{a-c}Means in the same section within a parameter with different superscripts differ ($P < 0.05$).

¹CTR = control diets; CT10 = 10 g of CT/kg of diet; CT30 = 30 g of CT/kg of diet; CO25 = 25 g of CO/kg of concentrate; CT10CO25 = 10 g of CT/kg of diet + 25 g of CO/kg of concentrate; CT30CO25 = 30 g of CT/kg of diet + 25 g of CO/kg of concentrate.

Fibrobacter succinogenes, *R. flavefaciens*, and *R. albus* have been considered representative cellulolytic bacterial species in the rumen (Koike and Kobayashi, 2009). In the present study, addition of CO reduced ($P < 0.05$) the *F. succinogenes* population. No CO effects were observed on fungi, *R. flavefaciens*, or *R. albus* populations. These results are in agreement with the in vitro observations of Kongmun et al. (2011), who observed that supplementation of CO reduced *F. succinogenes* population, but did not affect fungi, *R. flavefaciens*, or *R. albus* populations in rumen fluid obtained from swamp buffaloes. Similar results were observed in an in vivo experiment by Kongmun et al. (2011), who found that swamp buffaloes fed diets containing 7% CO had lower *F. succinogenes* population than those fed the control diet. Two possible reasons explain this finding: first, CO prevented digesta attachment of *F. succinogenes* by coating rumen digesta as it is known that *F. succinogenes* are found in rumen digesta more than in rumen fluid (Wanapat and Cherdthong, 2009); second, the effect of CO on rumen ciliate protozoa might indirectly affect the extent, type, and metabolic activity of the other rumen microbes, including *F. succinogenes* (Machmüller and Kreuzer, 1999). In addition, although addition of CO reduced the *F. succinogenes* population in the present study, CO seemed to have no effect on ruminal fiber fermentability because no changes in molar proportions of VFA occurred.

CONCLUSIONS

The use of CT, CO, and CT + CO had no adverse effects on growth performance but reduced CH₄ emissions in sheep. Addition of CT reduced the NH₃-N concentration in rumen fluid in CT30. Addition of CO decreased the total VFA concentration in rumen fluid. Concerning rumen microbes, addition of CT, CO, and CT + CO decreased methanogen and protozoa populations. Moreover, the population of *F. succinogenes* was decreased by addition of CO. Our results demonstrate that CT plus CO had an additive effect on inhibiting CH₄ emissions. Further research needs to be carried out to identify the long-term effects of CT and CO on CH₄ emission and rumen microbial populations.

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