Difference in the nature of tannins on in vitro ruminal methane and volatile fatty acid production and on methanogenic archaea and protozoal populations

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ABSTRACT

Six plant sources of hydrolyzable tannins (HT) or HT and condensed tannins (CT; designated as HT1, HT2, HT3, HT + CT1, HT + CT2, and HT + CT3) were evaluated to determine their effects in vitro on CH₄ production and on ruminal archaeal and protozoa populations, and to assess potential differences in biological activities between sources containing HT only or HT and CT. Samples HT1, HT2, and HT3 contained only HT, whereas samples HT + CT1, HT + CT2, and HT + CT3 contained HT and CT. In experiment 1, in vitro incubations with samples containing HT or HT + CT resulted in a decrease in CH₄ production of 0.6 and 5.5%, respectively, compared with that produced by incubations containing the added tannin binder polyethylene glycol-6000. Tannin also suppressed the population of methanogenic archaea in all incubations except those with HT2, with an average decrease of 11.6% in HT incubations (15.8, 7.09, and 12.0 in HT1, HT2, and HT3) and 28.6% in incubations containing HT + CT (35.0, 40.1, and 10.8 in HT + CT1, HT + CT2, and HT + CT3) when compared with incubations containing added polyethylene glycol-6000. The mean decrease in protozoal counts was 12.3% in HT and 36.2% in HT + CT incubations. Tannins increased in vitro pH, reduced total VFA concentrations, increased propionate concentrations, and decreased concentrations of iso-acids. In experiment 2, when a basal diet was incubated with graded levels of HT + CT1, HT + CT2, and HT + CT3, the total gas and CH₄ production and archaeal and protozoal populations decreased as the concentration of tannins increased. Our results confirm that tannins suppress methanogenesis by reducing methanogenic populations in the rumen either directly or by reducing the protozoal population, thereby reducing methanogens symbiotically associated with the protozoal population. In addition, tannin sources contain-

ing both HT and CT were more potent in suppressing methanogenesis than those containing only HT.

Key words: tannin, methane, methanogenic archaea, protozoon

INTRODUCTION

Ruminal methanogenic organisms use hydrogen produced during carbohydrate fermentation to reduce CO₂ to CH₄, thereby maintaining low partial pressures of hydrogen, which allows the oxidation of reduced NAD (Schonhusein et al., 2003). Despite this beneficial role in the rumen microbial ecosystem, the production of CH₄ is an energetically wasteful process to ruminants (Anderson et al., 2003). Methane emission by ruminants has received considerable attention because of its contribution to global warming (Lassey, 2007). Therefore, CH₄ reduction strategies should improve ruminant production efficiency and mitigate global warming.

Direct ruminal intervention is a means to control ruminant CH₄ emissions (Joblin, 1999), because CH₄-producing archaea, known as methanogens, are a distinct group of organisms that form a normal component of the rumen microbial ecosystem (Tavendale et al., 2005). Hydrogen and CO₂ are the major substrates for ruminal methanogens, and compounds that inhibit the activity of methanogens are likely to reduce or eliminate CH₄ production. Based on their structure and chemical properties, tannins are divided into hydrolyzable tannins [**HT**, which have a central carbohydrate core to which number of phenolic carboxylic acids are bound by esters of gallic acid (gallotannin) or ellagic acid (ellagitannins)] and condensed tannins (**CT**, or proanthocyanidines, which have no carbohydrate core and are derived by condensation of flavonoid precursors or polymers of flavonoids; Baker, 1999). Although tannins are generally regarded as antinutritional, certain tannins at low concentrations alter ruminal fermentation (Bhatta et al., 2002) and microbial protein synthesis (Bhatta et al., 2001). Tannins also reduce ruminal CH₄ production when included either as temperate legumes (Waghorn et al., 2002) or as purified tannin extracts

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Table 1. Nutrient composition of the TMR, hydrolyzable tannins (HT), or hydrolyzable and condensed tannins (HT + CT)¹

Item	HT ²			HT + CT ³			TMR ⁴
	HT1	HT2	HT3	HT + CT1	HT + CT2	HT + CT3	
DM	88.6	90.9	90.9	88.7	93.4	90.5	87.5
OM (g/kg of DM)	98.6	98.7	95.7	92.2	98.2	91.0	95.9
Ash (g/kg of DM)	1.40	1.29	4.35	7.80	1.80	9.00	4.07
CP (g/kg of DM)	1.02	2.39	1.42	0.710	2.13	1.10	13.1
NDF (g/kg of DM)	4.03	3.98	4.07	4.06	3.86	5.06	41.6
ADF (g/kg of DM)	2.01	1.96	2.14	1.78	1.85	2.67	24.2
Gross energy (Mcal/kg of DM)	3.70	3.78	3.56	4.45	4.78	4.37	4.04
Tannin concentration (% of DM)							
HT	13.0	13.2	18.5	3.94	7.62	7.78	—
CT	—	—	—	1.33	3.67	1.50	—

¹HT = hydrolyzable tannin as gallotannin; CT = condensed tannin as leucocyanidin equivalent.

²HT1, HT2, and HT3 = samples containing only HT (HT1 from myrabolam; HT2 and HT3 from chestnut).

³HT + CT1, HT + CT2, and HT + CT3 = samples containing HT plus CT (HT + CT1 and HT + CT2 from quebracho; HT + CT3 from mimosa).

⁴TMR contained 65% timothy hay, 20% crushed corn, and 15% soybean meal.

(Roth et al., 2002). However, there are no reports on potential differences in the activities of HT and CT on CH₄ production and on methanogenic archaeal and ciliated protozoal populations. The present study was conducted to determine the effects of plant materials containing different tannins (HT or HT + CT) on in vitro CH₄ production and on ruminal archaeal and ciliated protozoal populations, and to determine the difference, if any, in tannin sources containing HT only or both HT and CT.

MATERIALS AND METHODS

Source of Tannins

Six commercially available natural sources of tannins (designated as HT1, HT2, HT3, HT + CT1, HT + CT2, and HT + CT3) were used in this study. According to information obtained from the supplier (Kawamura and Co. Ltd., Asakusabashi, Taito-ku, Tokyo, Japan), HT1 (130 g of HT/kg of DM) was an extract from myrabolam, HT2 (132 g of HT/kg of DM) and HT3 (185 g of HT/kg of DM) were from chestnut, HT + CT1 (39.4 g of HT/kg of DM + 13.3 g of CT/kg of DM) and HT + CT2 (76.2 g of HT/kg of DM + 36.7 g of CT/kg of DM) were from quebracho, and HT + CT3 (77.8 g of HT/kg of DM + 15.0 g of CT/kg of DM) was from mimosa (Table 1). The products were supplied in the form of a fine dry powder.

Tannin Estimation

A 0.1-g sample of each tannin source was extracted with 10 mL of 70% (vol/vol) aqueous acetone in a 50-mL stoppered Erlenmeyer flask for 20 h at room tem-

perature. After centrifugation at $2,795 \times g$ for 15 min, the supernatant was made up to 10 mL with butanol HCl in the presence of iron, using rhodanine reagent (Makkar, 2003). Condensed tannin was expressed as leucocyanidin equivalent and hydrolyzable tannin as gallotannin (Makkar, 2003).

In Vitro Gas Production

In vitro gas production was determined by the procedure of Menke and Steingass (1988). In experiment 1 (with tannin-containing samples only), the effects of the different tannin-containing samples on in vitro methanogenesis was assessed by incubating samples with and without addition of a tannin binder, polyethylene glycol (**PEG**; PEG-6000, Wako Pure Chemical Industries Ltd., Osaka, Japan; 400 mg). Tannin-containing samples (200 mg) were weighed into 100-mL calibrated glass syringes (Häberle Labortechnik, Ettlenschief, Germany) with pistons lubricated with Vaseline. Buffered mineral solution (Menke and Steingass, 1988) was prepared and placed in a water bath at 39°C under continuous flushing with CO₂. Rumen fluid was collected before the morning feeding from 3 ruminally cannulated, non-lactating Holstein cows (466 kg of mean BW) fed 5.8 kg of timothy hay, 1.6 kg of crushed corn, and 0.80 kg of soybean meal. Ruminal contents were collected into a prewarmed insulated flask, transported to the laboratory, homogenized, and filtered through 3 layers of cheesecloth. An anaerobic condition was maintained by continuous flushing with CO₂. The well-mixed and CO₂-flushed, strained ruminal fluid was added to the buffered mineral solution (1:2). The mixture was kept stirred with a magnetic stirrer under CO₂ in a water bath at 39°C. A mixture of ruminal fluid and buffer

(30 mL) was dispensed into each syringe containing the weighed tannin samples. After closing the clips on the silicon tube at the syringe tip, syringes were gently shaken and clips were opened to remove gas by pushing the piston upward to achieve complete gas removal. The clip was closed, the initial volume was recorded, and the syringes were immediately placed in a thermostatically controlled shaking water bath at 39°C. Incubation of each tannin sample was set up in triplicate, and incubations were repeated 3 times, with intervals of 1 wk to obtain 9 observations for each feed sample. Incubations without a feed sample served as blanks. A parallel incubation of reference standard feedstuffs (hay and concentrate) obtained from the Hohenheim University (Hohenheim, Germany), as suggested by Menke et al. (1979), was also included. The gas produced after 24 h was recorded and gas samples were collected in evacuated vials and analyzed for CH₄.

In experiment 2 (tannin-containing samples added to a basal diet), 200 mg of DM of a basal diet consisting of timothy hay (65%), crushed corn (20%), and soybean meal (15%) was incubated as described in experiment 1 with different levels of HT + CT1, HT + CT2, and HT + CT3. Samples containing CT were chosen because of the significant ($P < 0.001$) reduction in CH₄ production observed in experiment 1. The tannin samples were included at levels of 0, 5, 10, 15, 20, and 25% of the basal diet DM.

CH₄ 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 (Polapack type Q, 80/100 mesh, Waters Associates Inc., Milford, MA), and a C-R3A integrator (Shimadzu). The column temperature was maintained at 50°C and Ar (0.5 kg/cm²) was used as the carrier gas. A mixture of H (1.04%), CH₄ (22.8%), CO₂ (33.6%), and N₂ (42.5%) served as the standard (Kajikawa et al., 2007).

Nutrient Analysis

Basal diet and tannin-containing samples were analyzed for DM, total N, ether extract, ash (AOAC, 1990), NDF (Van Soest et al., 1991), and ADF (Robertson and Van Soest, 1981). The NDF was analyzed in diet samples without sodium sulfite (with sodium sulfite in tannin samples) and with the use of a heat stable-amylase. Both NDF and ADF were expressed with residual ash. Gross energy content of samples was determined in an adiabatic bomb calorimeter (CA-4 PJ, Shimadzu).

Rumen Fluid Analysis

Volatile fatty acids were determined (Tajima et al., 2007) by gas chromatography (6890 series gas chromatograph with a flame-ionization detector, Hewlett-Packard, Wilmington, DE) on a glass column with 5% Thermon 1000 and 0.5% H₃PO₄ on 80/100 mesh Chromosorb W (Wako Pure Chemical Ltd., Osaka, Japan). Ammonia-N was determined colorimetrically (Tajima et al., 2007) with a Technicon Auto Analyzer II (Shimadzu).

In Situ DM Digestibility

Diet samples were ground to pass through a 2-mm screen and were mixed with the required amount of tannin-containing materials. Five grams of each sample was weighed, placed into a nylon bag (made of polyester monofilament, 53 µm pore size, 10 × 20 cm bag), and incubated in 2 ruminally cannulated, nonlactating Holstein cows (466 kg of mean BW) fed 5.8 kg of timothy hay, 1.6 kg of crushed corn, and 0.80 kg of soybean meal with vitamin and mineral supplements. After a 48-h in situ incubation, bags were removed and immediately placed in cold water to stop fermentation and to remove feed particles adhering to the bags. Subsequently, bags were placed in a domestic washing machine and washed for 20 min in water at 22 to 25°C. Bags were dried to a constant weight at 65°C, cooled to room temperature in desiccators, and weighed to determine DM disappearance.

Quantification of Archaeal and Bacterial 16S rRNA

Ruminal fluid was clarified according to Whitford et al. (1998) and total RNA was extracted by the bead-beating method (Krause and Russell, 1996). After a 24-h in vitro incubation (experiments 1 and 2), rumen fluid samples were collected and total RNA was extracted with phenol equilibrated to pH 5.1 with a buffer (10 mM EDTA, 50 mM sodium acetate, pH 5.1; Uyeno et al., 2004). Deoxyribonucleic acid contamination was removed by treating samples with RNase-free DNase (Promega, Madison, WI). Total RNA was stored at -80°C until analysis.

rRNA Quantification

The following probes (Uyeno et al., 2007) were used for the detection of bacterial and archaeal 16S rRNA: Arc915 (CCCCCGCCAATTCCTTTA; formamide concentration, 30%; cleavage coefficient, 0.80) and Eub338 [a mixture of equal amounts of 3 probes (GCTGCCTCCGTAGGAGT, GCAGCCACCCGTAGGTGT, and

GCTGCCACCCGTAGGTGT); formamide concentration, 20%; cleavage coefficient, 0.96]. The sequence-specific cleavage of rRNA fragments was performed as described previously (Uyeno et al., 2004). Briefly, 10 μ L of RNA solution (100 ng/ μ L), 5 μ L of 15 $\times$ hybridization buffer [375 mM Tris-HCl (pH 7.5), 15 mM EDTA, 375 mM NaCl], 2 μ L of oligonucleotide probe solution (10 pmol/ μ L), and a defined amount of formamide were mixed to make a hybridization solution, and diethyl pyrocarbonate-treated water was added to make a final volume of 75 μ L. The mixture was subsequently heated at 95°C for 1 min to unfold the RNA molecules, and the mixture was kept at the hybridization-digestion temperature (50°C) for 1 min. To initiate the cleavage reaction, 25 μ L of preheated 4 $\times$ enzyme solution [25 mM Tris (pH 7.5), 40 mM MgCl₂, 25 mM NaCl, 4 mM dithiothreitol, 120 mg of BSA/mL, 20 U/ μ L of cloned Ribonuclease H from *Escherichia coli* (TaKaRa, Kyoto, Japan)] was added to the mixture. After incubation at 50°C for 15 min, 50 μ L of stop solution [30 mM EDTA, 0.9 M sodium acetate (pH 7.0)] was added to the mixture to terminate the reaction. The RNA in the mixture was purified by ethanol precipitation and subjected to electrophoresis by an Agilent 2100 bioanalyzer with an RNA 6000 nano kit (Agilent, Palo Alto, CA).

Signal intensities of the respective peaks in the electrophoresed bands were determined and converted to peak areas by the Biosizing software associated with the device. The percentage of cleaved small subunit (SSU) rRNA was calculated using the following equation: percentage of cleaved SSU rRNA in the total SSU rRNA = (a + b)/(a + b + c) $\times$ 100, where a and b are the respective peak areas of fragmented SSU rRNA derived from the cleavage, and c is the intact (i.e., non-fragmented) SSU rRNA. The percentage was converted to the SSU rRNA population of the target group in total SSU rRNA by the following calculation: (SSU rRNA population of the target group) = (percentage of cleaved SSU rRNA)/(cleavage coefficient of the scissor probe). This equation measured values that were corrected with a coefficient to estimate the actual values of the population.

Enumeration of Ciliated Protozoa

After terminating the in vitro incubations of experiments 1 and 2, 1 mL of rumen fluid was diluted with 4 mL of saline containing formalin and 0.03% methyl green. Total ciliated protozoa in samples were counted using a Fuchs-Rosenthal chamber (0.2 mm depth, 2 $\times$ 2 mm chamber, 0.25 mm square lined) under 10 $\times$ magnification in a microscope (type BH-2, Olympus Corporation, Tokyo, Japan; Ogimoto and Imai, 1981).

Statistical Analysis

Data [NH₃-N, total VFA and CH₄ concentrations, 16S rRNA-based archaeal and bacterial populations, ciliated protozoal counts, and in vitro DM digestibility (IVDMD)] of tannin samples with and without PEG-6000 and the basal diet incubated with different levels of tannin samples were analyzed by the GLM procedure, using SAS/STAT version 9.1 (SAS Institute, 2004). The general model used was $Y_{ij} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \epsilon_{ij}$, in which Y_{ij} is the dependent variable, μ is the least squares mean, α_i is the effect of tannins, β_j is the effect of the PEG-6000, $\alpha\beta_{ij}$ is the effect of the interaction of tannins and PEG-6000, and ϵ_{ij} is the residual error. Treatment means were compared using Tukey's multiple comparisons procedure, and effects were considered significant at a probability value of $P < 0.05$.

RESULTS

Nutrient composition and tannin contents of samples are presented in Table 1. All samples had low CP, with the highest amount in HT2 (2.4% of DM). Contents of both NDF and ADF were very low in all samples, possibly because the commercial samples used were in a fine powder; hence, considerable amounts were filtered through the crucible after detergent treatment. Gross energy was greater than 4.10 Mcal/kg in all samples. Total tannin (HT + CT) contents (% of DM) in HT + CT1, HT + CT2, and HT + CT3 were 5.3, 11.3, and 9.3, respectively. The TMR contained 13.1% CP and 4.04 Mcal of gross energy. The NDF and ADF were 41.6 and 24.2 (% of DM), respectively. Tannin samples HT + CT1, HT + CT2, and HT + CT3, incubated at 0, 5, 10, 15, 20, and 25% of the basal diet, provided total tannin concentrations (CT + HT) of 0, 0.27, 0.53, 0.79, 1.06, and 1.32% in the HT + CT1 incubation; 0, 0.57, 1.13, 1.70, 2.26, and 2.83% in the HT + CT2 incubation; and 0, 0.47, 0.93, 1.38, 1.85, and 2.33% in the HT + CT3 incubation.

Total Gas Production, CH₄ Production, and Rumen Microbial Population In Vitro

Total gas production was lower ($P < 0.001$) in incubations containing tannin than in incubations containing tannin plus PEG-6000 (Table 2), and the difference was greater ($P < 0.001$) in HT + CT than in HT incubations. When PEG-6000 was added to incubations containing only HT, gas production averaged approximately 5 mL/200 mg of DM, compared with 9.0, 13.5, and 9.0 mL/200 mg of DM for HT + CT1, HT + CT2, and

Table 2. In vitro total gas and CH₄ production from tannin samples¹ with and without polyethylene glycol (PEG)²

Item	HT ³						HT + CT ⁴						SEM
	HT1	HT1 + PEG	HT2	HT2 + PEG	HT3	HT3 + PEG	HT + CT1	HT + CT1 + PEG	HT + CT2	HT + CT2 + PEG	HT + CT3	HT + CT3 + PEG	
24-h gas production (mL/200 mg of DM)	18.5 ^{de}	24.3 ^b	18.2 ^e	23.6 ^b	20.7 ^c	25.7 ^a	0.7 ^h	9.5 ^f	6.1 ^g	19.6 ^{cd}	0.5 ^h	9.5 ^f	0.216
CH ₄ (% of total gas)	13.5 ^{bc}	13.5 ^{bc}	13.4 ^{bc}	12.5 ^c	8.76 ^d	9.33 ^d	15.1 ^b	20.2 ^a	14.7 ^b	19.7 ^a	13.8 ^{bc}	20.3 ^a	0.328
CH ₄ (mM/g of DM)	0.57 ^c	0.75 ^b	0.56 ^c	0.69 ^b	0.42 ^d	0.55 ^c	0.02 ^f	0.43 ^d	0.20 ^e	0.86 ^a	0.02 ^f	0.43 ^d	0.01

^{a-h}Means within a row without a common superscript letter differ ($P < 0.001$).

¹HT = hydrolyzable tannin as gallotannin; CT = condensed tannin as leucocyanidin equivalent.

²Tannin sample and PEG (PEG-6000; Wako Pure Chemical Industries Ltd., Osaka, Japan) were incubated at a 1:2 ratio (wt/wt).

³HT1, HT2, and HT3 = samples containing only HT (HT1 from myrabolam; HT2 and HT3 from chestnut).

⁴HT + CT1, HT + CT2, and HT + CT3 = samples containing HT plus CT (HT + CT1 and HT + CT2 from quebracho; HT + CT3 from mimosa).

Table 3. Effects on ruminal microbial population of hydrolyzable tannins (HT) or hydrolyzable and condensed tannins (HT + CT)¹ with or without polyethylene glycol (PEG) addition²

Item	HT ³						HT + CT ⁴						SEM
	HT1	HT1 + PEG	HT2	HT2 + PEG	HT3	HT3 + PEG	HT + CT1	HT + CT1 + PEG	HT + CT2	HT + CT2 + PEG	HT + CT3	HT + CT3 + PEG	
Archaea (% of 16S rRNA)	9.4 ^e	11.1 ^{cd}	11.8 ^{bcd}	12.7 ^{ab}	11.0 ^d	12.5 ^{ab}	8.0 ^f	12.3 ^{bcd}	7.0 ^f	11.8 ^{bcd}	12.4 ^{bc}	13.9 ^a	0.27
Bacteria (% of 16S rRNA)	80.2 ^{ab}	80.1 ^{ab}	81.2 ^{ab}	81.1 ^{ab}	76.9 ^{bc}	70.2 ^d	51.4 ^e	33.4 ^g	81.8 ^{ab}	71.1 ^{cd}	73.1 ^{cd}	42.2 ^f	1.2
Total protozoa ($\times 10,000$ /mL)	5.27 ^b	5.90 ^{ab}	4.18 ^d	4.78 ^c	5.23 ^b	6.06 ^a	2.38 ^g	2.70 ^f	1.80 ^h	3.24 ^e	1.60 ⁱ	3.36 ^e	1.0

^{a-i}Means within a row without a common superscript letter differ ($P < 0.001$).

¹HT = hydrolyzable tannin as gallotannin; CT = condensed tannin as leucocyanidin equivalent.

²Tannin samples and PEG (PEG-6000; Wako Pure Chemical Industries Ltd., Osaka, Japan) were incubated at a 1:2 ratio (wt/wt).

³HT1, HT2, and HT3 = samples containing only HT (HT1 from myrabolam; HT2 and HT3 from chestnut).

⁴HT + CT1, HT + CT2, and HT + CT3 = samples containing HT plus CT (HT + CT1 and HT + CT2 from quebracho; HT + CT3 from mimosa).

HT + CT3, respectively (Table 2). Incubations with HT + CT1, HT + CT2, and HT + CT3 produced 5.1, 5.0, and 6.5% less CH₄ ($P < 0.001$) than incubations containing added PEG-6000 (Table 2). On average, decreases in CH₄ output were 0.6 and 5.5% in the HT and HT + CT incubations, respectively, when compared with incubations containing added PEG-6000. When expressed as mM per gram of DM, the HT1, HT2, and HT3 incubations produced 23.6, 18.7, and 23.8% less CH₄ ($P < 0.05$) and the HT + CT1, HT + CT2, and HT + CT3 incubations produced 94.4, 76.9, and 85.8% less CH₄ ($P < 0.001$) than their respective incubations containing tannin plus PEG-6000.

Tannin suppressed ($P < 0.001$) the populations of methanogenic archaea in all incubations except those containing HT2, compared with the PEG-6000-supplemented incubations. The average decrease was approximately 11.6% in the HT incubations (15.8, 7.09, and 12.0% in the HT1, HT2, and HT3 incubations, respectively), compared with 28.6% in the HT + CT incubations (35.0, 40.1, and 10.8 in HT + CT1, HT + CT2, and HT + CT3, respectively; Table 3). There was an increase of only 2.9% in the total bacterial population in the HT incubations (0.1, 0.1, and 8.7% in HT1, HT2, and HT3, respectively), whereas it was 30.0% ($P < 0.001$) in the HT + CT incubations (35.0, 13.0, and 42.3% in HT + CT1, HT + CT2, and HT + CT3, respectively; Table 3). Incubations containing tannin had reduced total ciliated protozoal counts compared with incubations with added PEG-6000. The mean decrease in ciliated protozoal numbers was 12.3% in HT samples and was 36.2% in HT + CT samples.

***In Vitro* pH, Gas Production, NH₃-N and VFA Concentrations, and IVDMD**

The final pH and the NH₃-N (mg/dL), total VFA (mM), and individual VFA (mM) concentrations from incubations with and without PEG-6000 addition are shown in Table 4. Tannins increased pH, reduced ($P < 0.001$) VFA concentrations, and tended to increase the molar proportion of propionate compared with the PEG-6000-supplemented incubations. The proportion of butyrate was not affected. Concentrations of iso-acids decreased ($P < 0.001$) in the presence of tannin.

When the basal diet (no tannin) was incubated with incremental levels of HT + CT1, HT + CT2, and HT + CT3, total gas production, CH₄ output (mM/g of DM incubated), and archaea and protozoal populations decreased as the concentration of tannin increased (Tables 5, 6, and 7), except in incubations with HT + CT3. The magnitude of the change varied at lower levels (up to 10%) among 3 tannin sources; however, at 25% inclusion, CH₄ production (1.05 mM/g of DM) was

similar among all samples. In contrast to PEG-6000 addition to tannin samples, the total bacterial population decreased when the tannin concentration increased in the HT + CT1 and HT + CT3 incubations, but no specific trend was observed in the HT + CT2 incubations. There was a decrease in CH₄ concentration up to 10% tannin inclusion in HT + CT1, HT + CT2, and HT + CT3, and at higher levels the concentration plateaued. In vitro DM digestibility indicated no change at lower levels of tannin inclusion; however, at higher levels (>15%), DM digestibility decreased ($P < 0.001$), except in the HT + CT3 incubation.

The correlations between total tannin (% of TMR), archaea population (% of total bacteria), and CH₄ output (mM/g of DM) are presented in Table 8. There was a negative correlation between total tannins and the archaea population and CH₄ output, indicating that as tannin concentration increased, the archaea population and CH₄ output decreased. The correlation was highest ($r = 0.90$) in HT + CT2 compared with HT + CT1 ($r = 0.85$) and HT + CT3 ($r = 0.42$).

The effect of graded levels of HT + CT1, HT + CT2, and HT + CT3 in the basal diet on NH₃-N, pH, and VFA are presented in Tables 5, 6, and 7, respectively. Increasing the tannin level resulted in reduced NH₃-N and VFA concentrations. There was a decrease in acetic acid and an increase in propionic acid concentration with tannin supplementation. The concentration of iso-acids also decreased when tannin concentration increased.

DISCUSSION

Samples containing HT + CT were more effective in reducing total gas and CH₄ production than samples containing HT only. The effects of HT + CT on total gas and CH₄ production were greater compared with HT. In an earlier study, we showed that in vitro incubations with tamarind (*Tamarindus indica*) seed husk (containing approximately 140 g of CT/kg of DM), had lower gas production compared with incubations that had the added tannin-binder PEG (Bhatta et al., 2001). Waghorn et al. (2002) reported that in vitro incubation of *Lotus pedunculatus* (containing tannin) had lower CH₄ production compared with *L. pedunculatus* treated with PEG. In the same study, sheep fed *L. pedunculatus* produced 17% less CH₄ compared with sheep fed *L. pedunculatus* plus PEG. Very few reports are available regarding the biological activities of tannin fractions on ruminal CH₄ production. Extracts of HT, such as gallotannic acid, showed 50% inhibition of CH₄ production, with lower inhibition associated with monomers compared with polymers (Field and Lettinga, 1987). Further studies are needed with fraction-

Table 4. In vitro pH, NH₃-N (mg/dL), and VFA production (mM) in fermentations of tannin samples¹ with and without polyethylene glycol (PEG)²

Sample	HT ³						HT + CT ⁴						SEM
	HT1	HT1 + PEG	HT2	HT2 + PEG	HT3	HT3 + PEG	HT + CT1	HT + CT1 + PEG	HT + CT2	HT + CT2 + PEG	HT + CT3	HT + CT3 + PEG	
pH	6.72 ^a	6.59 ^{ab}	6.73 ^a	6.65 ^a	6.44 ^{bc}	6.03 ^d	6.68 ^a	6.58 ^{ab}	6.68 ^{ab}	6.62 ^{ab}	6.37 ^c	6.31 ^c	0.039
NH ₃ -N	3.93 ^{cde}	4.83 ^b	4.08 ^{cd}	4.98 ^b	3.75 ^e	3.84 ^{de}	4.02 ^{cd}	5.07 ^b	3.18 ^f	5.46 ^a	4.14 ^c	5.37 ^a	0.055
Total VFA ⁵	46.3 ^c	83.5 ^a	47.4 ^c	78.5 ^{ab}	45.0 ^c	76.9 ^{ab}	43.0 ^c	79.1 ^{ab}	48.1 ^c	77.2 ^{ab}	41.8 ^c	72.3 ^b	1.397
C2	31.7 ^d	60.0 ^a	32.7 ^d	58.0 ^{ab}	31.5 ^d	55.7 ^{bc}	30.9 ^d	57.4 ^{ab}	31.8 ^d	56.8 ^{abc}	29.8 ^d	53.0 ^c	0.780
C3	8.3 ^{def}	14.9 ^a	8.6 ^{cdef}	14.3 ^a	7.4 ^f	10.5 ^{bcd}	7.5 ^{ef}	11.1 ^{bc}	9.6 ^{cdef}	13.1 ^{ab}	7.1 ^f	10.1 ^{cde}	0.512
C2:C3	3.85 ^{bc}	4.03 ^b	3.78 ^{bc}	4.05 ^b	4.27 ^b	5.30 ^a	4.12 ^b	5.18 ^a	3.30 ^c	4.34 ^b	4.17 ^b	5.27 ^a	0.132
C4	5.1 ^{bcd}	6.0 ^{ab}	5.1 ^{bcd}	4.9 ^{cd}	4.35 ^d	5.7 ^{abc}	4.1 ^d	6.3 ^a	6.0 ^{ab}	5.5 ^{abc}	4.1 ^d	5.5 ^{abc}	0.202
C5	0 ^b	0.7 ^{ab}	0.2 ^b	0.4 ^{ab}	0.4 ^{ab}	0.9 ^a	0 ^b	0.5 ^{ab}	0 ^b	0.7 ^{ab}	0 ^b	0.4 ^{ab}	0.138
iC4 + iC5	1.2 ^c	2.0 ^{bc}	0.7 ^c	1.0 ^c	1.6 ^c	4.2 ^a	0.5 ^c	3.9 ^a	0.7 ^c	1.2 ^c	0.6 ^c	3.3 ^{ab}	0.339

^{a-f}Means within a row without a common superscript letter differ ($P < 0.001$).

¹HT = hydrolyzable tannin as gallotannin; CT = condensed tannin as leucocyanidin equivalent.

²Tannin sample and PEG (PEG-6000; Wako Pure Chemical Industries Ltd., Osaka, Japan) were incubated at a 1:2 ratio (wt/wt).

³HT1, HT2, and HT3 = samples containing only HT (HT1 from myrabolam; HT2 and HT3 from chestnut).

⁴HT + CT1, HT + CT2, HT + CT3 = samples containing HT plus CT (HT + CT1 and HT + CT2 from quebracho; HT + CT3 from mimosa).

⁵C2 = acetic acid; C3 = propionic acid; C4 = butyric acid; C5 = valeric acid; iC4 + iC5 = isobutyric + isovaleric acids.

ated extracts from the tannin samples to identify the specific component(s) responsible for antimethanogenic activity.

Reports on the effects of HT and CT on ruminal ciliated protozoal counts are somewhat conflicting. Extracts containing HT were reported to have no significant effect on protozoal counts (Sliwinski et al., 2002), whereas Leinmuller et al. (1991) reported HT as less inhibitory than CT on protozoal numbers. However, Terrill et al. (1992) did not observe any adverse effect of CT-containing sulla (*Hadysarum coronarium*) on protozoal numbers. In our study, differences in protozoal numbers observed between tannin-containing samples and tannin plus PEG-6000 incubations confirmed that the effects of HT and CT on protozoal populations were not similar, with HT + CT exhibiting greater antiprotozoal activity than HT only. This difference in the effects of HT + CT- and HT-containing tannin samples on protozoal populations likely contributed to the difference in antimethanogenic activity observed in our incubations. Protozoa contribute hydrogen for the reduction of CO₂ to CH₄ by the methanogens. Methane production is higher when protozoa are present or present in greater numbers in the rumen than when they are absent or present in low numbers (Jouany and Lassalas, 1997).

Tannins decreased in vitro NH₃-N concentrations in all incubations compared with PEG-6000-supplemented incubations; however, the magnitude of decrease was greater in HT + CT incubations than in HT incubations alone (Table 4). A similar trend was also observed

when the basal diet was incubated with increased levels of samples containing HT + CT. This can be attributed to the formation of complexes between HT and CT with proteins. The HT and proteins usually form complexes at an optimal pH range of 3 to 4, but complexes also occur in appreciable amounts under typical rumen pH of 6 to 7 (Leinmuller and Menke, 1990). Some ruminal bacteria can dissociate the protein-HT complexes, thus indicating that the formation of these complexes is at least partially reversible. However, the dissociation of protein-CT complexes is difficult (McSweeney et al., 2001). The observed difference in NH₃-N concentrations between HT and HT + CT may be attributable to the reversible nature of the protein-HT complex.

In the absence of tannin (or when bound with PEG-6000), the degradability of protein was higher, resulting in a greater NH₃-N concentration, possibly because of inhibition of microbial deaminase by tannins (Leinmuller and Menke, 1990). When sheep were changed from a diet of perennial rye grass and white clover (which did not contain CT) to a diet of *Lotus corniculatus* containing CT (32 g of CT/kg of DM), the population of ruminal proteolytic *Butyrivibrio fibrisolvens* decreased. When PEG was infused into the rumen, populations of those proteolytic bacteria increased significantly (Min et al., 2003). Low NH₃-N was also reported in sheep (Bhatta et al., 2007) and goats (Bhatta et al., 2002, 2005) consuming tannin-containing *Prosopis cineraria* leaves (10 g of CT/kg of DM). Ewes grazing the CT-containing forage *L. corniculatus* showed lower ruminal NH₃ concentrations than ewes grazing CT-free forage

Table 5. Effects of hydrolyzable plus condensed tannin (HT + CT1) on in vitro CH₄ production and rumen microbial population

Item	Tannin concentration ¹ (% of TMR, ² DM basis)						SEM	<i>P</i> -value
	0	5	10	15	20	25		
24-h gas production (mL/200 mg of DM)	40.8 ^a	39.0 ^{ab}	38.0 ^{ab}	36.0 ^{bc}	34.8 ^{bc}	32.7 ^c	0.920	0.0005
CH ₄								
% of total gas	17.7	17.0	16.2	17.0	16.9	17.2	0.324	0.1317
mM/g of DM	1.70 ^a	1.48 ^b	1.31 ^{bc}	1.25 ^{cd}	1.17 ^{cd}	1.05 ^d	0.043	0.0001
Archaeal population (% of 16S rRNA)	12.5 ^a	12.6 ^a	8.3 ^b	9.3 ^b	9.7 ^b	8.7 ^b	0.397	0.0001
Bacterial population (% of 16S rRNA)	70.8 ^a	71.9 ^a	69.0 ^a	68.4 ^a	66.7 ^b	64.2 ^c	1.220	0.0001
Protozoal population (×10,000/mL)	9.28 ^a	8.56 ^b	7.38 ^c	6.09 ^c	6.21 ^c	6.17 ^c	0.347	0.0001
IVDMD ³	0.665 ^a	0.664 ^a	0.655 ^a	0.651 ^a	0.640 ^b	0.630 ^b	0.009	0.0005
Fermentation product ⁴								
pH	6.32	6.32	6.35	6.37	6.34	6.33	0.022	0.556
NH ₃ -N (mg/dL)	8.18 ^a	7.27 ^b	6.85 ^{bc}	6.71 ^{bc}	6.65 ^{bc}	6.36 ^c	0.170	0.001
Total VFA (mM)	66.7 ^a	65.4 ^{ab}	63.0 ^{abc}	62.4 ^{abc}	60.6 ^{bc}	57.4 ^c	1.209	0.002
C2 (mM)	43.8 ^a	43.4 ^a	41.5 ^{ab}	41.5 ^{ab}	40.2 ^{ab}	38.0 ^b	0.767	0.002
C3 (mM)	12.2 ^a	12.1 ^a	11.8 ^{ab}	11.8 ^{ab}	11.4 ^{ab}	10.8 ^b	0.229	0.010
C2:C3	3.59	3.59	3.52	3.52	3.53	3.52	0.026	0.140
C4 (mM)	7.5 ^a	7.00 ^{ab}	7.0 ^b	6.8 ^{bc}	6.5 ^{bc}	6.3 ^c	0.107	0.001
C5 (mM)	1.00 ^a	0.81 ^{abc}	0.81 ^{abc}	0.66 ^c	0.87 ^{ab}	0.74 ^{bc}	0.043	0.002
iC4 + iC5 (mM)	2.32 ^a	2.11 ^{ab}	1.93 ^{bc}	1.76 ^{cd}	1.66 ^{cd}	1.56 ^d	0.062	0.001
CH ₄ :total VFA	39.2 ^a	44.2 ^b	48.1 ^c	49.9 ^d	51.8 ^e	54.7 ^f	0.072	0.002

^{a-f}Means within a row without a common superscript letter differ ($P < 0.001$).

¹0, 5, 10, 15, 20, and 25 = level of tannin sample containing hydrolyzable and condensed tannin (HT + CT1 from quebracho) as a percentage of TMR on a DM basis (wt/wt).

²TMR contained 65 parts timothy hay, 20 parts crushed corn, and 15 parts soybean meal.

³IVDMD = in vitro DM digestibility after 48 h.

⁴C2 = acetic acid; C3 = propionic acid; C4 = butyric acid; C5 = valeric acid; iC4+ iC5 = isobutyric + isovaleric acids.

Table 6. Effects of hydrolyzable plus condensed tannin (HT + CT2) on in vitro CH₄ production, rumen microbial population, and fermentation products

Item	Tannin concentration ¹ (% of TMR, ² DM basis)						SEM	<i>P</i> -value
	0	5	10	15	20	25		
24-h gas production (mL/200 mg of DM)	43.2 ^a	40.8 ^b	37.2 ^c	34.0 ^d	30.8 ^e	30.5 ^e	0.462	0.001
CH ₄								
% of total gas	18.8	18.2	16.2	17.1	17.2	17.2	0.919	0.445
mM/g of DM	1.90 ^a	1.67 ^{ab}	1.31 ^{bc}	1.19 ^c	1.05 ^c	1.04 ^c	0.075	0.001
Archaeal population (% of 16S rRNA)	12.0 ^a	9.6 ^b	8.7 ^{bc}	8.8 ^{bc}	8.8 ^{bc}	7.8 ^c	0.333	0.001
Bacterial population (% of 16S rRNA)	68.4 ^{cd}	69.4 ^{bc}	66.0 ^d	72.0 ^b	67.8 ^{cd}	78.0 ^a	0.656	0.001
Protozoal population (×10,000/mL)	9.28 ^a	8.91 ^b	7.81 ^c	6.33 ^c	6.64 ^c	4.14 ^c	0.584	0.001
IVDMD ³	0.665 ^a	0.651 ^a	0.650 ^a	0.642 ^b	0.630 ^b	0.625 ^c	0.007	0.004
Fermentation product ⁴								
pH	6.32 ^b	6.38 ^{ab}	6.32 ^b	6.39 ^{ab}	6.43 ^a	6.44 ^a	0.019	0.555
NH ₃ -N (mg/dL)	8.18 ^a	6.63 ^b	6.13 ^{bc}	5.75 ^{cd}	5.40 ^d	5.53 ^{cd}	0.127	0.001
Total VFA (mM)	66.7 ^a	64.1 ^{ab}	61.6 ^{bc}	59.1 ^{cd}	57.1 ^d	56.1 ^d	0.647	0.002
C2 (mM)	43.8 ^a	42.0 ^b	40.6 ^{bc}	39.4 ^{cd}	38.0 ^{de}	37.6 ^c	0.359	0.002
C3 (mM)	12.2 ^a	12.0 ^a	11.7 ^a	11.0 ^b	10.4 ^c	10.2 ^c	0.140	0.010
C2:C3	3.59 ^{ab}	3.50 ^b	3.47 ^b	3.58 ^{ab}	3.65 ^a	3.68 ^a	0.035	0.140
C4 (mM)	7.48 ^a	7.16 ^a	6.56 ^b	6.36 ^b	6.24 ^b	6.22 ^b	0.083	0.001
C5 (mM)	1.00 ^a	0.83 ^a	0.94 ^a	0.65 ^b	0.78 ^a	0.69 ^b	0.088	0.002
iC4+ iC5 (mM)	2.32 ^a	2.10 ^b	1.81 ^c	1.65 ^d	1.70 ^{cd}	1.76 ^{cd}	0.034	0.001
CH ₄ :total VFA	35.1 ^a	38.4 ^b	47.0 ^c	49.7 ^d	54.4 ^e	53.9 ^f	0.069	0.002

^{a-f}Means within a row without a common superscript letter differ ($P < 0.001$).

¹0, 5, 10, 15, 20, and 25 = level of tannin sample containing hydrolyzable and condensed tannin (HT + CT2 from quebracho) as a percentage of TMR on a DM basis (wt/wt).

²TMR contained 65 parts timothy hay, 20 parts crushed corn, and 15 parts soybean meal.

³IVDMD = in vitro DM digestibility after 48 h.

⁴C2 = acetic acid; C3 = propionic acid; C4 = butyric acid; C5 = valeric acid; iC4+ iC5 = isobutyric + isovaleric acids.

Table 7. Effects of hydrolyzable plus condensed tannin (HT + CT3) on in vitro CH₄ production, rumen microbial population, and fermentation products

Item	Tannin concentration ¹ (% of TMR, ² DM basis)						SEM	P-value
	0	5	10	15	20	25		
24-h gas production (mL/200 mg of DM)	42.3 ^a	40.3 ^b	41.0 ^b	39.0 ^c	38.2 ^c	36.0 ^d	0.236	0.001
CH ₄								
% of total gas	17.5 ^a	17.3 ^a	17.1 ^a	15.9 ^{ab}	15.9 ^{ab}	15.2 ^b	0.373	0.006
mM/g of DM	1.75 ^a	1.56 ^b	1.50 ^b	1.27 ^c	1.19 ^c	1.04 ^d	0.032	0.001
Archaeal population (% of 16S rRNA)	12.5	12.0	12.4	12.4	11.2	12.2	0.279	0.041
Bacterial population (% of 16S rRNA)	72.6 ^a	74.4 ^a	69.9 ^a	68.0 ^a	65.1 ^b	65.7 ^b	1.104	0.001
Protozoal population (×10,000/mL)	9.29 ^b	10.0 ^b	6.76 ^d	11.7 ^a	7.85 ^{cd}	8.48 ^c	0.637	0.001
IVDMD ³	0.665 ^a	0.659 ^a	0.656 ^a	0.650 ^a	0.646 ^a	0.628 ^b	0.011	0.001
Fermentation product ⁴								
pH	6.28 ^{ab}	6.25 ^b	6.32 ^{ab}	6.29 ^{ab}	6.31 ^{ab}	6.33 ^a	0.016	0.038
NH ₃ -N (mg/dL)	8.48 ^a	7.96 ^b	7.34 ^c	6.90 ^d	6.62 ^{de}	6.36 ^e	0.074	0.001
Total VFA (mM)	66.7 ^a	66.8 ^a	62.8 ^{ab}	63.4 ^{ab}	62.9 ^{ab}	61.6 ^b	0.933	0.007
C2 (mM)	43.8 ^a	43.5 ^{ab}	40.9 ^c	41.3 ^{bc}	41.0 ^c	40.2 ^c	0.482	0.001
C3 (mM)	12.2	12.5	11.6	11.8	11.8	11.9	0.225	0.132
C2:C3	3.59 ^a	3.48 ^{ab}	3.53 ^{ab}	3.50 ^{ab}	3.47 ^{ab}	3.38 ^b	0.040	0.050
C4 (mM)	7.48	7.66	7.33	7.35	7.26	7.17	0.115	0.200
C5 (mM)	1.00	0.97	0.92	0.95	0.89	0.89	0.123	0.277
iC4+ iC5 (mM)	2.32 ^a	2.28 ^a	2.10 ^b	2.06 ^b	2.03 ^{bc}	1.89 ^c	0.035	0.001
CH ₄ :total VFA	38.1 ^a	42.8 ^b	41.9 ^c	49.9 ^d	52.9 ^e	59.2 ^f	0.086	0.003

^{a-f}Means within a row without a common superscript letter differ ($P < 0.001$).

¹0, 5, 10, 15, 20, and 25 = level of tannin sample containing hydrolyzable and condensed tannin (HT + CT3 from mimosa) as a percentage of TMR on a DM basis (wt/wt).

²TMR contained 65 parts timothy hay, 20 parts crushed corn, and 15 parts soybean meal.

³IVDMD = in vitro DM digestibility after 48 h.

⁴C2 = acetic acid; C3 = propionic acid; C4 = butyric acid; C5 = valeric acid; iC4+ iC5 = isobutyric + isovaleric acids.

(Min et al., 1998). The significant decrease in the NH₃-N concentration may be attributed to inhibition of the bacteria-degrading activity of protozoa (Jouany, 1994).

Total bacterial counts were greater ($P < 0.001$) and protozoal counts were lower (except for HT3) in the presence of tannin (no PEG-6000). The reduction in concentrations of iso-acids further supports our findings. Iso-acids are derived from AA catabolism in the rumen (Mackie and White, 1990). Concentrations of iso-acids were lower in the presence of tannin because the protein was protected from ruminal deamination. Results were also similar when the basal diet (without tannin) was incubated with graded levels HT + CT. The maximum concentration of iso-acids was reported with nontanniferous legumes, whereas lower concentrations were found in legumes with high CT (Tavendale et al., 2005). In our study, reductions in iso-acids were recorded only in HT + CT, but not in HT, again reflecting the difference in the type of tannins.

The reduction in pH after PEG-6000 addition was due to greater VFA production. Tannins tended to increase the molar proportion of propionate. A similar trend was observed when the basal diet (no tannin) was incubated with graded levels of tannin. Lower propionate production in the presence of PEG has also been reported (Nuñez-Hernandez et al., 1991). Production of methane

and propionate is negatively correlated because of the competition for hydrogen. Additionally, enhanced propionate production is associated with an increase in the flow of microbial protein from the rumen (Tavendale et al., 2005). Protozoa were responsible for the shift in VFA proportions toward acetic and butyric acids. Further, the transfer of hydrogen to archaea is facilitated by a synergistic interaction between methanogens and ciliates (Vogels et al., 1980). The positive relationship between the acetic acid:propionic acid ratio and CH₄ output is also clearly evident from our results.

Samples containing HT + CT exhibited greater antimethanogenic activity than HT alone, whereas the effects on other ruminal microbes appeared less severe. Our results suggest that reduced CH₄ production with tannin was primarily due to its antimethanogenic activ-

Table 8. Correlation between total tannins¹ (% of TMR, DM basis), archaeal population, and CH₄ output

Item	HT + CT1	HT + CT2	HT + CT3
Total tannins:archaeal population	-0.758	-0.860	-0.430
Total tannins:CH ₄ output	-0.976	-0.954	-0.991
Archaeal population:CH ₄ output	0.849	0.903	0.419

¹HT + CT1, HT + CT2, and HT + CT3 = samples containing hydrolyzable plus condensed tannin (HT + CT; HT + CT1 and HT + CT2 from quebracho; HT + CT3 from mimosa).

ity, and not solely because of reduced fermentation of OM, as reported in earlier studies. Ultee et al. (2002) noted that compounds with phenolic structures were more effective as antimicrobials in comparison with other nonphenolic secondary plant metabolites because of the presence of a hydroxyl group in the phenolic structure. Methanogenic archaea are associated symbiotically with the ciliate protozoa on the surface (ectosymbionts) and inside the protozoa (endosymbionts; Finlay et al., 1994); hence, a reduction in protozoal counts may decrease archaeal counts as well. In the presence of tannin, both protozoal and archaeal numbers were reduced, which likely led to a compensatory increase in the population of other ruminal bacteria (Jouany, 1994). However, Soliva et al. (2004) did not find an increase in the bacterial population after suppression of protozoa, and this was attributed to an adverse effect of tannins on some bacterial species as well. In our second experiment, in which graded levels of the tannin source (HT + CT) were incubated with the basal diet, there was no increase in bacterial counts despite the suppression of protozoa. A similar observation was made by Soliva et al. (2004).

When the basal diet was incubated with graded levels of HT + CT1, HT + CT2, and HT + CT3, the total gas and CH₄ produced and the archaeal and protozoal populations decreased. There was a decline in CH₄ output from the 0 to 10% level of HT + CT1 and HT + CT2 samples and thereafter a plateau, whereas in HT + CT3, the decrease was gradual. Hayler et al. (1998) reported that the amount of CH₄ produced per unit of fermented substrate *in vitro* was lower as the level of tannin-containing feed in the mixture increased. Differences in CH₄ production among samples were attributed to variations in content of tannins and their biological activity. The IVDMD values reflected an adverse effect of tannins only at greater levels. This further confirmed that the suppression of CH₄ recorded at a lower level of tannins was primarily due to the antimethanogenic and antiprotozoal activities of the tannins, leading to the decrease in methanogenic archaea counts, but at greater levels, it was likely due to the combined effect of decreased ruminal microbes (archaea + protozoa) and decreased OM fermentation. The ratios of CH₄ to total VFA depicted in Tables 5, 6, and 7 confirm this finding.

A suitable compound for inhibition of methanogenesis in meat-producing animals would be one that is effective in reducing CH₄ production and one that also increases propionate (Tavendale et al., 2005). The tannin sources tested in our studies appear to have those attributes for use in ruminant feeding to suppress methanogenesis. However, it must be noted that the long-term effects might be different because adaptation

of the rumen microbes might occur. Therefore, long-term feeding trials in animals need to be carried out to determine their effects on animal response and also to observe changes in the rumen microbial population.

CONCLUSIONS

Our investigation confirmed that tannins suppress methanogenesis directly through their antimethanogenic activity and indirectly through their antiprotozoal activity. Samples containing HT + CT were more potent in reducing methanogenesis than those containing HT only. Incubation of the basal diet (no tannin) with graded levels of the tannin source showed a linear response on CH₄ suppression. Further studies need to be carried out to identify the specific chemical component responsible for and mechanism of action of antimethanogenic activity. In addition, animal feeding studies are needed to determine the long-term effects on rumen microbial populations and animal performance.

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