Effects of high condensed-tannin substrate, prior dietary tannin exposure, antimicrobial inclusion, and animal species on fermentation parameters following a 48 h in vitro incubation¹

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ABSTRACT: Condensed tannins (CT), prior dietary CT exposure, animal species, and antimicrobial inclusion effects on 48 h extent of in vitro fermentation were measured in an experiment with a 3 × 2 × 2 × 3 factorial arrangement of treatments. Treatments included species of inoculum donor (*Bos taurus*, *Ovis aries*, or *Capra hircus*; $n = 3/\text{species}$), prior adaptation to dietary CT (not adapted or adapted), culture substrate (low-CT or high-CT), and antimicrobial additive (none, bacterial suppression with penicillin + streptomycin, or fungal suppression with cycloheximide). Low-CT or high-CT substrates were incubated in vitro using inoculum from animals either not exposed (period 1) or previously exposed to dietary CT (period 2). The extent of IVDMD after 48 h of incubation was greater ($P < 0.001$) for cultures with low-CT substrate (21.5%) than for cultures with

high-CT substrate (16.5%). Cultures with high-CT substrate or with suppressed bacterial activity had less ($P < 0.001$) gas pressure than cultures with low-CT substrate or cultures with suppressed fungal activity. Total VFA concentrations were greater ($P < 0.001$) in low-CT cultures when inoculum donors were without prior CT exposure (83.7 mM) than when inoculum was from CT-exposed animals (79.6 mM). Conversely, total VFA concentrations were greater ($P < 0.001$) in high-CT cultures with tannin-exposed inoculum (59.4 mM) than with nonexposed inoculum (52.6 mM). As expected, CT and suppression of bacterial fermentative activities had strong negative effects on fermentation; however, prior exposure to dietary CT attenuated some negative effects of dietary CT on fermentation. In our experiment, the magnitude of inoculum-donor species effects on fermentation was minor.

Key words: adaptation, condensed tannins, in vitro fermentation

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INTRODUCTION

Condensed tannins (CT) are polymers of flavonoid units with a high capacity to bind to proteins and affect enzymatic activity (Waghorn,

2008). They are found in a wide variety of plants and are a functional defense mechanism against diseases, stress, and herbivory (Min et al., 2003). CT limit DMI, DM digestibility, and ruminal protein degradation by ruminants due to formation of CT-protein complexes in vivo (Makkar, 2003; Eckerle et al., 2011); CT-protein complexes are formed during mastication when CT are released from plant cells (Min et al., 2003). These complexes are stable under ruminal conditions and render proteins largely undegradable by microbial

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enzymes (Makkar, 2003). Ruminal protein degradation decreased with the addition of CT in vitro (Hassanat and Benchaar, 2012) and in vivo (Al-Dobaib, 2009). Hassanat and Benchaar (2012) demonstrated a decrease in total VFA concentrations and in butyrate, valerate, and branched-chain VFA molar proportions in the presence of CT. Min et al. (2005) reported that CT suppressed growth rates in 11 selected species of ruminal bacteria.

Small ruminants reportedly have greater tolerance for high-tannin forages than beef cattle. Frutos et al. (2004) reported that in vitro gas production and DM disappearance were greater for goats and deer than for cattle and sheep when CT were included in culture media. Little research has focused on adaptability of various ruminant species to dietary CT. In addition, the relative susceptibilities of ruminal fungi and ruminal bacteria to dietary CT are unknown. Therefore, our objective was to evaluate simultaneously the influences of prior exposure to dietary CT in *Bos taurus*, *Ovis aries*, and *Capra hircus*, with and without selective antimicrobial suppression of either ruminal bacteria or ruminal fungi on IVDMD, gas production, ammonia concentrations, and concentrations of total and individual VFA in 48 h in vitro fermentation cultures.

MATERIALS AND METHODS

The Kansas State University Institutional Animal Care and Use Committee reviewed and approved all animal handling and animal care practices used in our experiment. All animal procedures were conducted in accordance with the Guide for the Care and Use of Animals in Agricultural Research and Teaching (FASS, 2010).

This in vitro experiment evaluated two factors applied to inoculum donors as well as two factors that were applied within fermentation vessels. The experiment used a $3 \times 2 \times 2 \times 3$ factorial arrangement of treatments. Treatments relevant to the inoculum donors included species (*Bos taurus*, *Ovis aries*, or *Capra hircus*) and prior adaptation to dietary CT (not adapted or adapted). Treatments applied to the fermentation vessels included substrate (low-CT or high-CT) and antimicrobial treatment (none, bacterial suppression with penicillin + streptomycin, or fungal suppression with cycloheximide).

The experimental design was a split-split plot, with the main plot arranged as a randomized block with the treatment being species of inoculum donor (cow, sheep, or goat) and block being the cohort

group (i.e., one animal from each species). The subplot treatment was dietary adaptation of inoculum donors to CT, which was confounded with period. Within each subplot (i.e., each animal within each period), treatments applied randomly to the in vitro vessels in a 2×3 factorial arrangement were culture substrate and antimicrobial additive.

Inoculum Donors

Three beef cows (551 ± 30 kg BW), three sheep (68 ± 3 kg BW), and three goats (49 ± 4 kg BW) were used. Sheep and goats were housed together in a 10×10 m pen and cows were housed in an adjacent 100×100 m pen. Smooth brome grass hay (*Bromus inermis*; 87.9% DM; DM composition = 9.1% CP, 67.0% NDF, and 41.3% ADF) was offered to all animals daily in round-bale feeders (diameter = 2.5 m) in amounts calculated to exceed ad libitum intake ($\approx 4\%$ BW/d). Smooth brome grass hay had no detectable CT, as determined according to Makkar (2003).

One animal (i.e., inoculum donor) from each species was assigned randomly to 1 of 3 cohorts; cohorts were assigned randomly to one of three sampling times during each of two experimental periods. Animals were fed a common low-CT diet during period 1 and a common high-CT diet during period 2.

All animal cohorts were fed smooth brome grass hay for ad libitum intake throughout period 1. All animals also had continual, unrestricted access to fresh water, a salt block (98.0% NaCl; Compass Minerals, Chicago, IL), and a mineral block (95.5% NaCl, 3,500 ppm Zn, 2,000 ppm Fe, 1,800 ppm Mn, 280 ppm Cu, 100 ppm I, and 60 ppm Co; Compass Minerals). Ruminal fluid for in vitro batch cultures was collected via stomach tube from cohort 1 on d 11, from cohort 2 on d 14, and from cohort 3 on d 17.

During period 2, animals were adapted to high-CT intake by providing them with grain-by-product supplements that were spiked with quebracho tannins (QT). Animals were fed tannin-free smooth brome grass hay for ad libitum intake, and each animal was also individually fed a supplement that contained QT extract at 0.1% of BW/d, soybean hulls at 0.2% of BW/d, and dried molasses at 0.05% of BW/d. Purified, feed-grade QT was procured from Wintersun Chemical (Ontario, CA) for use in the high-CT supplements. Soybean hulls and molasses were fed to encourage complete consumption of the prescribed dose of QT. The animals were individually penned each morning

(0730 h) and each evening (2000 h) with supplement and fresh water available. They were allowed access to the supplement for 45 min at each feeding; any unconsumed supplement following the morning feeding was offered again during the evening feeding. Unconsumed supplement following the evening feeding was collected and weighed to determine intake. Daily consumption of CT averaged 2.6 ± 0.16 g/kg BW^{0.75}, 2.9 ± 0.10 g/kg BW^{0.75}, and 3.2 ± 0.13 g/kg BW^{0.75} for goats, sheep, and cattle, respectively. In period 2, all animals had continual access to fresh water, and the same salt block and mineral block as in period 1. Ruminal fluid for in vitro batch cultures was collected via stomach tube from cohort 1 on d 22, from cohort 2 on d 25, and from cohort 3 on d 28 of period 2.

Ruminal Fluid Collection

Ruminal fluid was collected orally at 0730 h on the schedule designated for each animal cohort. A simple vacuum strainer was constructed for this purpose. Briefly, a cylindrical coarse particle filter (10 × 1 cm) made of copper and drilled throughout with 5 cm holes was fitted to the distal end of a polyethylene tube (300 × 1 cm). The proximal end of the polyethylene tube was connected to a vacuum bottle which, in turn, was connected to an electrical vacuum pump. The intervening vacuum bottle was used to prevent fluids from inadvertently being drawn into the pump.

Ruminal fluid was collected from each animal in a cohort in the following order: cattle, sheep, and goat. Cattle were restrained using a chute with a locking head gate. Sheep and goats were restrained manually. An oral speculum was inserted into the mouth (15 cm for cattle and 8 cm for sheep and goats) to prevent animals from severing the collection tube. The collection tube, detached from the vacuum pump, was inserted filter-side first into the esophagus. Once the tube reached the cardia, air was blown gently into it to force the cardia open and allow passage of the tube into the rumen. The tube was then attached to the pump. To prevent excessive salivary contamination, the first 200 mL of ruminal fluid collected from each animal was discarded; an additional 700 mL of fluid were harvested and retained at each collection. Collection time was approximately 7 min/animal.

Harvested ruminal fluid from each animal was transferred immediately to a single 3 L, prewarmed, insulated bottle for transport to laboratory facilities. Harvested ruminal fluid was strained through eight layers of cheesecloth and poured into a separatory

flask; it was allowed to settle in the flask for 30 min at 39 °C. The heaviest fraction, containing protozoa, feed particles, and fungal sporangium associated with feed particles, was discarded.

In Vitro Substrates

During periods 1 and 2, the low-CT substrate added to ruminal inoculum of cattle, sheep, and goats was smooth brome grass hay (87.9% DM; DM composition = 9.1% CP, 67.0% NDF, and 41.3% ADF). Hay was ground (#4 Wiley Mill, Thomas Scientific, Swedesboro, NJ) to pass a 1 mm screen before it was added to culturing devices. Ground smooth brome grass hay (3.067 g, DM basis) was added to culture jars assigned to low-CT substrate treatments.

The high-CT substrate added to ruminal inoculum of cattle, sheep, and goats during periods 1 and 2 was a composite of the smooth brome grass hay used as the low-CT substrate and CT in the form of QT. Condensed-tannin concentration in QT (71.8%, DM basis) was determined by the Friedberg Skin-Powder method (Wintersun Chemical, Ontario, CA). Composition of QT was 92.4% DM, 1.3% CP, 0.4% NDF, and 2.5% ADF (DM basis). Smooth brome grass hay (2.637 g, DM basis) and QT (0.43 g, DM basis) were added to culture jars assigned to high-CT substrate treatments. This mixture achieved a calculated substrate CT concentration of 10.1% (DM basis).

Antimicrobial Treatments

To determine the relative importance of bacterial and fungal fermentative activities, in vitro fermentations were subject to the addition of antimicrobial compounds to the culture ([Windham and Akin, 1984](#)). Bacterial suppression was accomplished using penicillin G (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co, Milwaukee, WI) and streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co); a bacterial-suppression solution was prepared containing 12.5 mg of penicillin G and 2 mg of streptomycin sulfate per mL of deionized water. Fungal suppression was accomplished by preparing a solution of 5 mg of cycloheximide (C7698; Sigma-Aldrich Chemical Co) per mL of deionized water.

In Vitro Fermentations

In vitro mean gas pressure and concentrations of ammonia and VFA were measured using a 48 h

batch-culture technique. Cultures were incubated using 250 mL glass jars equipped with ANKOM gas production system lids (Model RF1; ANKOM Technology, Macedon, NY). For each inoculum source (i.e., each individual animal within a cohort in each period), six jars received one of six substrate $\times$ antimicrobial treatment combinations and three jars (blanks) received no substrate but did receive one of the three antimicrobial treatments (i.e., each antimicrobial treatment was represented in a substrate-free blank). Each fermentation jar received in sequence 3.067 g of either low-CT substrate or high-CT substrate described previously (DM basis; no substrate was added to jars designated as blanks), 5 mL of deionized water or antimicrobial solution, 50 mL of ruminal fluid, and 100 mL of McDougall's artificial saliva. McDougall's artificial saliva was prepared 24 h before each ruminal fluid collection and stored in an incubator at 39 °C (McDougall, 1948). Nitrogen gas was bubbled through the McDougall's artificial saliva for a minimum of 30 min before it was added to fermentation vessels; during this time, the pH was adjusted to between 6.8 and 7.0 using aqueous metaphosphoric acid (25 g of HPO_3 in 100 mL deionized water).

Jars were individually gassed with N_2 for 20 s and capped with an ANKOM RF1 lid. The jars were placed in an orbital-shaking incubator (Model G25; New Brunswick Scientific Co, Inc, New Brunswick, NJ) in random order at 39 °C and agitated lightly for 48 h. After 48 h, jars were removed and immediately placed on ice to suppress fermentation.

Gas pressure in the headspace of each jar was recorded every 15 min during the 48 h incubation. Average gas pressures for the 48 h period were reported. Samples for VFA and ammonia analyses were collected by transferring 1 mL of fluid from each jar, in duplicate, into separate 2 mL conical vials, into which 0.25 mL of aqueous metaphosphoric acid (25%, wt/vol) had been added to suspend microbial activity. Samples were frozen (−20 °C) pending analysis.

Concentrations of VFA were measured via GLC as described by Mullins et al. (2010). Before analyses, samples were thawed for 30 min, vortexed for 10 s, and centrifuged at $16,000 \times g$ for 10 min (Eppendorf model 5415C; Eppendorf North America, Hauppauge, NY). Ammonia concentrations were analyzed as described by Broderick and Kang (1980) using a Technicon Autoanalyzer II (Technicon Industrial Systems, Tarrytown, NY).

In vitro rate of digestibility was measured using a 48 h time-series, culture technique.

Cultures were conducted using 50 mL plastic centrifuge tubes sealed with rubber stoppers. Stoppers were equipped with a venting tube that allowed fermentation gasses to escape but excluded atmospheric air. Centrifuge tubes were dried at 105 °C for 3 h prior to use; tubes were cooled in a desiccator and weighed individually to determine dry weight. In vitro DM disappearance was measured after incubation periods of 4, 8, 12, 24, 36, and 48 h.

Each treatment was represented by six tubes (one/time point); moreover, 18 blank tubes per animal species (i.e., containing ruminal inoculum but no substrate; three/time point, one for each antimicrobial treatment) were incubated during each period. At 24 h before ruminal fluid collection, either the low-CT or high-CT substrates described previously were added to each tube, excepting those designated as blanks. Tubes designated to receive low-CT substrate were dosed with 0.5 g of smooth bromegrass hay (DM basis), whereas tubes designated to receive high-CT substrate were dosed with 0.43 g smooth bromegrass hay and 0.07 g QT (DM basis). This mixture achieved a substrate CT concentration similar (i.e., 10.1% of DM) to that used in our batch-culture system.

Penicillin-streptomycin sulfate solution (1 mL) and cycloheximide solution (1 mL) were added to designated tubes; immediately thereafter, ruminal fluid (10 mL) from each species within a cohort was added to tubes using a tilting, repeating dispenser (Windham and Akin, 1984). McDougall's artificial saliva (20 mL) was also added to each tube at that time. Tubes were individually gassed with N_2 for 10 s, capped, and placed in a random order within tube racks by species and incubation end-time. All tubes were incubated at 39 °C.

At each incubation-time end point, one tube from each treatment $\times$ species combination ($n = 6$) and three blanks were removed from the incubator and placed on ice. Tubes were then placed in a centrifuge (Beckman J2-21; Beckman Coulter, Inc, Brea, CA) at $13,800 \times g$ for 15 min. Tubes, with residual substrate and fluid, were placed in a forced-air oven at 105 °C for 48 h; tubes with dry samples were placed in a desiccator to cool and were then weighed. In vitro DM disappearance was determined using procedures described by Smith et al. (2013).

Culture substrates were analyzed for partial DM (Goering and Van Soest, 1970), DM (Goering and Van Soest, 1970), N (AOAC, 2000; 968.06) using combustion analysis (Leco TruMac N, St. Joseph, MI), NDF (Van Soest et al., 1991;

modified for the Ankom 200 fiber analyzer, Ankom Technology Corp.), and ADF (AOAC, 2005; 973.18 modified for the Ankom 200 fiber analyzer, Ankom Technology Corp.).

Statistical Analyses

Blank-corrected mean gas pressures and concentrations of ammonia and VFA were analyzed as a split-split plot design using PROC MIXED of SAS (SAS Inst. Inc., Cary, NC). Fixed effects in the model included animal species (cow, sheep, or goat), adaptation of inoculum donors to tannins (not adapted or adapted), culture substrate (low-CT or high-CT), microbial suppressant (none, penicillin + streptomycin, or cycloheximide), and all possible 2-, 3-, and 4-way interaction terms. Random effects included animal cohort, cohort $\times$ species, and dietary tannin exposure within cohort $\times$ species.

Blank-corrected IVDMD was analyzed in the same manner as other fermentation indices; however, length of incubation (4, 8, 12, 24, 36, or 48 h) and a five-way interaction (animal species $\times$ adaptation $\times$ culture substrate $\times$ microbial suppressant $\times$ incubation time) were added as fixed effects.

Significance was declared at $P \leq 0.001$ due to the large number of interactions and our desire to avoid type 2 errors. When protected by a significant F -test ($P \leq 0.001$), means were separated by Least Significant Difference. Least-squares means for the highest order, significant ($P \leq 0.001$) interaction terms are reported.

RESULTS AND DISCUSSION

In Vitro DM Disappearance

The effects of culture substrate on IVDMD were influenced ($P < 0.001$) by incubation time (Figure 1). Cultures with low-CT substrate had greater ($P < 0.001$) IVDMD than cultures with the high-CT substrate after 24, 36, and 48 h of incubation. In vitro DM disappearance in cultures with low-CT and high-CT substrates generally increased between 4 and 36 h of incubation; however, IVDMD for both treatments did not change between 36 and 48 h of incubation. The extent of IVDMD after 48 h of incubation was roughly 8.5 percentage units greater for cultures with low-CT substrate than for cultures with high-CT substrate. Getachew et al. (2008) reported that batch cultures similar to ours that were dosed with high-CT substrates had approximately 17 percentage units less DM disappearance than cultures dosed with tannin-free substrates.

Mean Gas Pressure

Effects of culture substrate on mean gas pressure were influenced by donor-animal exposure to dietary tannins (substrate $\times$ tannin-exposure status, $P < 0.001$; Table 1). Mean gas pressures were greater ($P < 0.001$) in cultures assigned to low-CT substrate (1.74 bar) than for cultures assigned to high-CT substrate (0.88 bar); reduction in mean gas pressure due to CT was not

Figure 1. Effects of incubation time and substrate CT content on IVDMD during a 48 h incubation. Low-CT substrate was *Bromus inermis* hay only (0.5 g; DM basis), and high-CT substrate was *Bromus inermis* hay (0.4299 g; DM basis) and quebracho tannin (0.0701 g; DM basis), which provided 10.1% CT in the substrate. Within incubation-time point, separation of error bars indicates difference between substrate types ($P < 0.001$; LSD = 3.69). Within low-CT substrate, means not bearing a common superscript letter of a, b, or c differ between incubation-time points ($P < 0.001$; SEM = 2.15). Within high-CT substrate, means not bearing a common superscript letter of d, e, or f differ between incubation-time points ($P < 0.001$; SEM = 2.15).

alleviated by prior exposure to CT. Hassanat and Benchaar (2012) reported that gas production in cultures containing QT was reduced by doses ≥ 20 g QT/kg substrate. Tan et al. (2011) also demonstrated a dose-dependent decrease in total gas production with increasing QT. Makkar (2003) speculated that decreased gas production may be due to decreased fiber digestion in the presence of CT.

Effects of culture substrate on mean gas pressures also were influenced ($P < 0.0001$) by antibiotic addition to 48 h in vitro batch cultures (Table 2). Mean gas pressures were not different ($P < 0.001$) between antibiotic-free cultures given low-CT substrate and cycloheximide-spiked cultures given low-CT substrate (2.11 and 1.98 bar, respectively). In addition, mean gas pressures were not different between antibiotic-free cultures with high-CT

Table 1. Effects of substrate CT content and prior adaptation of inoculum donors to dietary CT on mean gas pressures and concentrations of ammonia, total VFA, and acetate following a 48 h in vitro incubation

Item	No CT adaptation ^a		Dietary CT adaptation ^b		SEM ^c
	Low-CT substrate ^c	High-CT substrate ^d	Low-CT substrate ^c	High-CT substrate ^d	
Mean gas pressure ^{f,g,h} , bar	1.83 ⁱ	0.82 ^j	1.65 ⁱ	0.94 ^j	0.641
Ammonia concentration ^{f,g,h} , mM	19.4 ^{ij}	18.5 ^{ijk}	19.9 ^j	16.7 ^k	0.70
Total VFA concentration ^{f,g,h} , mM	83.7 ⁱ	52.6 ^l	79.6 ^j	59.4 ^k	2.27
Acetate concentration ^{f,g,h} , mM	57.8 ⁱ	36.7 ^k	51.3 ^j	37.3 ^k	1.93

^aCultures were inoculated with ruminal fluid from *Bos taurus*, *Ovis aries*, and *Capra hircus* donors fed a CT-free diet.

^bCultures were inoculated with ruminal fluid from *Bos taurus*, *Ovis aries*, and *Capra hircus* donors fed a high-CT diet for 21 d.

^cCultures substrate consisted of ground *Bromus inermis* hay only (3.067 g; DM basis).

^dCultures substrate consisted of ground *Bromus inermis* hay (2.637 g; DM basis) and quebracho-tannin extract (0.43 g; DM basis), which created a substrate CT concentration of 10.1% (DM basis).

^eIn cases where observations between treatments were unequal, the largest SEM was reported.

^fMain effect of CT adaptation ($P \leq 0.001$).

^gMain effect of substrate ($P \leq 0.001$).

^hCT adaptation $\times$ substrate ($P \leq 0.001$).

^{i,j,k,l} Within row, means without a common superscript letter differ ($P < 0.001$).

Table 2. Effects of substrate CT content and antimicrobial additive on mean gas pressures and concentrations of total VFA, acetate, butyrate, valerate, and branched-chain VFA following a 48 h in vitro incubation

Item	No antimicrobial additive		Penicillin + streptomycin ^a		Cycloheximide ^b		SEM ^c
	Low-CT substrate ^c	High-CT substrate ^d	Low-CT substrate ^c	High-CT substrate ^d	Low-CT substrate ^c	High-CT substrate ^d	
Mean gas pressure ^{f,g,h} , bar	2.11 ^j	0.99 ^j	1.14 ^k	0.65 ^m	1.98 ^j	1.02 ^j	0.737
Total VFA concentration ^{f,g,h} , mM	96.1 ^j	63.6 ^j	57.2 ^m	41.7 ⁿ	91.6 ^k	62.7 ^j	2.08
Acetate ^{f,g,h} , mM	60.5 ^j	39.7 ^m	45.6 ^j	32.0 ⁿ	57.5 ^k	39.2 ^m	1.80
Butyrate ^{f,g,h} , mM	7.08 ^j	4.44 ^j	3.60 ^m	2.89 ⁿ	6.47 ^k	3.98 ^m	0.135
Valerate ^{f,g,h} , mM	0.79 ^j	0.44 ^k	0.22 ^j	0.24 ^j	0.75 ^j	0.42 ^k	0.041
Branched-chain VFA ^{f,g,h,i} , mM	1.55 ^j	0.76 ⁿ	1.05 ^j	0.73 ^{mn}	1.24 ^k	0.60 ⁿ	0.118

^aCultures contained 0.40 mg penicillin (1,600 U/mg; PEN-K; Sigma–Aldrich Chemical Co., Milwaukee, WI) and 0.064 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma–Aldrich Chemical Co.) per mL of culture to act as a suppressant to bacterial fermentative activities.

^bCultures contained 0.16 mg cycloheximide (C7698; Sigma–Aldrich Chemical Co.) per mL of culture to act as a suppressant to fungal fermentative activities.

^cCultures substrate consisted of ground *Bromus inermis* hay only (3.067 g; DM basis).

^dCultures substrate consisted of ground *Bromus inermis* hay (2.637 g; DM basis) and quebracho-tannin extract (0.43 g; DM basis), which created a substrate CT concentration of 10.1% (DM basis).

^eIn cases where observations between treatments were unequal, the largest SEM was reported.

^fMain effect of substrate ($P \leq 0.001$).

^gMain effect of antimicrobial additive ($P \leq 0.001$).

^hSubstrate $\times$ antimicrobial additive ($P \leq 0.001$).

ⁱCombined concentrations of isobutyrate and isovalerate.

^{j,k,l,m,n} Within row, means with unlike superscripts differ ($P < 0.001$).

substrate and cycloheximide-spiked cultures with high-CT substrate (0.99 and 1.02 bar, respectively). Thus, fermentative activities of ruminal fungi contributed little to total gas production in our 48 h batch culture in vitro system. Mean gas pressures in low-CT cultures that were spiked with penicillin + streptomycin (1.14 bar) were less ($P < 0.001$) than in low-CT cultures without antibiotic (2.11 bar) and low-CT cultures spiked with cycloheximide (1.98 bar). High-CT substrate also produced less gas when dosed with penicillin + streptomycin (0.65 bar) than when no antibiotic was added to high-CT cultures (0.99 bar) or when high-CT cultures were dosed with cycloheximide (1.02 bar). Clearly, suppression of bacterial fermentative activities had strong negative effects on gas production in our 48 h, batch culture in vitro system. Windham and Akin (1984) indicated that ruminal bacteria were responsible collectively for more fiber degradation than ruminal fungi, which agrees with our observations of mean gas pressure.

Ammonia Concentration

Ammonia concentrations were relatively high for all treatments because urea was added to the McDougal's artificial saliva used in our experiment. Ammonia concentration was influenced ($P < 0.001$) by both culture substrate and tannin-exposure status of ruminal fluid donors (Table 1). Concentration of ammonia was greatest ($P < 0.001$) in cultures given low-CT substrate, regardless of tannin-exposure status, (19.4 and 19.9 mM for nonexposed and exposed, respectively) and least in cultures given high-CT substrate and inoculated with ruminal fluid from tannin-exposed animals (16.7 mM). Ammonia concentrations in cultures with nontannin exposed ruminal inoculum and given a high-CT substrate were intermediate (18.5 mM). In each of these treatments, ammonia concentrations were sufficient to support maximal digestion rate of structural carbohydrates (Satter and Slyter, 1974); therefore, N availability should not have limited bacterial fermentative activities. Frutos et al. (2004) and Hassanat and Benchaar (2012) reported that ruminal ammonia concentrations were reduced in the presence of dietary CT due to decreased ruminal protein degradation. Therefore, we concluded that proteolytic activities of bacteria in high-CT cultures were likely inhibited in our experiment.

Total VFA Concentration

Total VFA concentrations in 48 h in vitro batch cultures were influenced ($P < 0.001$) by culture

substrate and tannin-exposure status of ruminal fluid donors (Table 1). Total VFA concentration was greatest ($P < 0.001$) when tannin-free medium was added to cultures inoculated with ruminal fluid from animals without prior exposure to dietary CT (83.7 mM); it was slightly less in cultures given tannin-free substrate and incubated with tannin-exposed inoculum (79.6 mM). Total VFA concentration decreased ($P < 0.001$) sharply in cultures with high-CT substrate; however, cultures with high-CT substrate and inoculated with tannin-exposed ruminal fluid had greater ($P < 0.001$) total VFA concentrations than cultures with high-CT substrate and inoculated with nonexposed ruminal fluid (59.4 and 52.6 mM, respectively). Culture substrates containing CT generally depressed total VFA concentration compared with cultures given tannin-free substrate. Prior exposure to dietary CT ameliorated this depression somewhat. CT have been noted to depress total VFA concentrations (Waghorn, 2008; Tan et al., 2011; Hassanat and Benchaar, 2012). We speculate that prior dietary exposure to CT partially alleviated detrimental effects of CT on total VFA concentration in ruminants by selecting for micro-organisms less sensitive to CT.

Effects of culture substrate on total VFA concentration were influenced ($P < 0.001$) by inclusion of antimicrobial additives in cultures (Table 2). Total VFA concentration was greatest ($P < 0.001$) in cultures with low-CT media and without antimicrobial additives (96.1 mM); it was slightly less for cultures with low-CT media and dosed with cycloheximide (91.6 mM). Both produced significantly greater VFA concentration at 48 h of incubation than high-CT substrate cultures without antimicrobial additive and cycloheximide-treated, high-CT cultures (63.6 and 62.7 mM, respectively). Cultures treated with penicillin + streptomycin produced less ($P < 0.001$) total VFA than other culture types. Within cultures treated with penicillin + streptomycin, low-CT substrate produced more ($P < 0.001$) total VFA than high-CT substrate (57.2 and 41.7 mM, respectively). Min et al. (2005) determined that CT concentrations $> 200 \mu\text{g CT/mL}$ of ruminal fluid reduced growth of 11 anaerobic bacterial strains. This may have exacerbated the effects of high-CT substrate and penicillin + streptomycin treatment in our study.

An interaction between antimicrobial treatment and tannin-exposure status of inoculum donors on total VFA was present ($P = 0.001$) because prior CT exposure led to numeric increases in total VFA concentrations for cultures without antimicrobial additive and for cultures dosed with cycloheximide,

whereas the opposite was true for cultures dosed with penicillin + streptomycin (Figure 2). Shifts in the microbial populations in response to prior CT exposure may have made microbial activities within cultures more susceptible to the antibacterial treatment. Because cycloheximide had little effect on overall fermentation, it is not surprising that cycloheximide-treated cultures demonstrated responses similar to cultures without antimicrobial additives when donor animals were exposed to CT.

Individual VFA Concentrations

An interaction between culture substrate and inoculum-donor species inoculum was detected ($P < 0.001$) for acetate concentration. *Bos taurus* and *Capra hircus* inoculum with low-CT substrate had the greatest ($P < 0.001$) acetate concentrations after 48 h of incubation; *Ovis aries* inoculum with low-CT substrate had less acetate (57.1, 55.0, and 51.6 mM, respectively; data not shown). Cultures with high-CT substrate had less acetate than cultures with tannin-free substrate (36.8, 35.8, and 38.4 mM for *Bos taurus*, *Ovis aries*, and *Capra hircus*, respectively), and there were no differences between inoculum-donor species. High-CT substrates depressed acetate concentrations approximately 30% across donor species compared with low-CT substrates. Tan et al. (2011) and Hassanat and Benchaar (2012) reported no change in acetate molar proportion in the presence of CT. We speculate that CT depressed fiber degradation in our experiment, resulting in lesser acetate concentrations.

Differences in tannin sensitivity between beef cattle and small ruminants are well documented

(Vaithiyathan et al., 2001; Frutos et al., 2004; Eckerle et al., 2010, 2011; Lamy et al., 2010; Pacheco et al., 2012; Lemmon et al., 2017). In our study, acetate concentrations following a 48 h in vitro incubation were mildly influenced by inoculum-donor species; however, no other fermentation index was affected. Thus, the effects of inoculum-donor species on fermentation in a batch-culture system, like those used in our study, were generally small compared with other factors we investigated (i.e., culture substrate, antimicrobial inclusion, and prior exposure to dietary CT).

An interaction for acetate concentration was detected between culture substrate and CT-exposure status of donor animals ($P < 0.001$; Table 1). In low-CT cultures, acetate concentrations were greater with no donor-animal exposure to CT than with prior donor-animal exposure to CT (57.8 and 51.3 mM, respectively). Apparently, prior CT exposure may have depressed acetate yield, even when CT were not present in the culture. Cultures with high-CT substrate contained the smallest acetate concentrations; however, there were no differences between them with respect to CT-exposure status of donor animals (36.7 and 37.3 mM for nonexposed and exposed, respectively).

Cultures with low-CT substrate had greater ($P < 0.001$) acetate concentrations than cultures with high-CT substrate, but acetate concentrations were also influenced ($P < 0.001$) by a culture substrate $\times$ antimicrobial treatment interaction (Table 2). In low-CT cultures, acetate concentrations were modestly decreased ($P < 0.001$) when cycloheximide was added to the cultures, whereas cultures dosed with penicillin + streptomycin

Figure 2. Effects of antimicrobial additive and prior dietary CT exposure on total VFA concentration following a 48 h in vitro incubation (LSD = 8.51; SEM = 2.329). Penicillin + streptomycin = 0.40 mg penicillin (1,600 U/mg) and 0.064 mg streptomycin sulfate (720 IU/mg) per mL of culture to act as a suppressant to bacterial fermentative activities. Cycloheximide = 0.16 mg cycloheximide per mL of culture to act as a suppressant to fungal fermentative activities. No prior CT exposure = inoculation with ruminal fluid from animals fed a tannin-free diet. Prior CT exposure = inoculation with ruminal fluid from animals fed a high-CT diet. Means not bearing a common superscript letter differ ($P = 0.001$).

had acetate concentrations that were strikingly decreased (60.5, 57.5, and 45.6 mM, for no additive, cycloheximide, and penicillin + streptomycin, respectively). Among cultures given high-CT substrates, those with no antimicrobial additive and those spiked with cycloheximide had marginally greater acetate concentrations than those spiked with penicillin + streptomycin (39.7, 39.2, and 32.0 mM, respectively), but the reduction associated with suppression of bacterial fermentative activities was less than that for cultures given low-CT substrate.

A three-way interaction was detected ($P < 0.001$) for propionate concentration following a 48 h in vitro incubation (Figure 3). Treatment with penicillin + streptomycin produced the smallest ($P < 0.001$) concentrations of propionate; substrate type and prior tannin exposure had no influence on propionate yield when bacterial activities were suppressed, reflecting the overwhelming effect of bacterial suppression. Propionate concentrations for cultures receiving either no antimicrobial or cycloheximide showed similar patterns; greater reductions in propionate were observed in response to CT being added to the substrate when inoculum was from animals not exposed to CT. In those cultures receiving no antimicrobial or cycloheximide, the negative effect of high-CT substrate on propionate concentrations was significantly ameliorated ($P < 0.001$) by the use of inoculum from animals exposed to CT. Tan et al. (2011) reported no change in propionate, and Hassanat and Benchaar (2012) reported a slight increase in propionate molar proportions in the presence of CT compared with

controls. In our experiment, propionate-producing organisms (most likely bacteria) received substantial benefit from prior exposure to dietary CT. In addition, suppression of fungal fermentative activities had little influence on propionate concentration following a 48 h in vitro incubation.

Across antimicrobial treatments, cultures with high-CT substrate had lesser ($P < 0.001$) concentrations of butyrate than cultures with tannin-free substrates, but effects of culture substrate on butyrate concentration were influenced ($P < 0.001$) by antimicrobial additive (Table 2). Within each substrate type (i.e., low-CT or high-CT), cultures not treated with an antimicrobial additive had the greatest ($P < 0.001$) butyrate concentrations (7.08 and 4.44 mM for low-CT and high-CT cultures, respectively); cycloheximide-spiked cultures were intermediate (6.47 and 3.98 mM for low-CT and high-CT cultures, respectively), and cultures spiked with penicillin + streptomycin had the least butyrate concentrations (3.60 and 2.89 mM for low-CT and high-CT cultures, respectively). Relative to antimicrobial-free cultures, butyrate concentrations were slightly reduced (<1 mM) by antifungal treatment and extensively reduced (1.5 to 3.5 mM) by antibacterial treatment. We interpret these data to indicate that both bacteria and fungi contributed to butyrate production; however, the magnitude of butyrate production by bacteria was larger than that of fungi. Reduction in butyrate molar proportion in the presence of high-CT substrate was reported by Getachew et al. (2008) and Hassanat and Benchaar (2012).

Figure 3. Effects of substrate CT content, antimicrobial additive, and prior dietary CT exposure on propionate concentration following a 48 h in vitro incubation (LSD = 3.7; SEM = 0.77). Low-CT substrate was *Bromus inermis* hay only (3.067 g; DM basis), and high-CT substrate was *Bromus inermis* hay (2.637 g; DM basis) and quebracho tannin (0.43 g; DM basis), which provided 10.1% CT in the substrate. Penicillin + streptomycin = 0.40 mg penicillin (1,600 U/mg) and 0.064 mg streptomycin sulfate (720 IU/mg) per mL of culture to act as a suppressant to bacterial fermentative activities. Cycloheximide = 0.16 mg cycloheximide per mL of culture to act as a suppressant to fungal fermentative activities. No prior CT exposure = inoculation with ruminal fluid from animals fed a tannin-free diet. Prior CT exposure = inoculation with ruminal fluid from animals fed a high-CT diet. Means not bearing a common superscript letter differ ($P < 0.001$).

Valerate concentrations were influenced ($P < 0.001$) by culture substrate and antimicrobial additive (Table 2). Within each substrate type, valerate concentrations were not different ($P < 0.001$) between antimicrobial-free cultures and cultures treated with cycloheximide; however, low-CT substrate was associated with greater valerate concentrations than high-CT substrate for these treatments (0.79 vs. 0.44 mM for antimicrobial-free cultures and 0.75 vs. 0.42 mM for cycloheximide-spiked cultures). Valerate concentrations were least ($P < 0.001$) in cultures treated with penicillin + streptomycin (0.22 and 0.24 mM for low-CT and high-CT cultures, respectively); substrate type did not influence valerate concentrations within penicillin + streptomycin-treated cultures. Similar responses to antimicrobial treatment and substrate type were noted with total VFA concentration in our study. Reductions in valerate molar proportion in the presence of high-CT substrates were reported also by Getachew et al. (2008) and Hassanat and Benchaar (2012). We conclude the ruminal fungal population did not contribute significantly to valerate concentration under the conditions of our study.

Concentrations of branched-chain VFA (isobutyrate + isovalerate; BCVFA) were influenced ($P < 0.001$) by culture substrate and antimicrobial treatment (Table 2). Within cultures given low-CT substrate, antimicrobial-free cultures had the greatest ($P < 0.001$) BCVFA concentrations; cultures treated with cycloheximide were intermediate, and cultures treated with penicillin + streptomycin had the least BCVFA concentrations (1.55, 1.24, and 1.05 mM, respectively). Cultures with high-CT substrate had lesser ($P < 0.001$) BCVFA than cultures with low-CT substrate. Antimicrobial-free cultures given high-CT substrate had greater ($P < 0.001$) BCVFA than cycloheximide-treated cultures given high-CT substrate; high-CT cultures treated with penicillin + streptomycin had intermediate BCVFA concentration and were not different from either antimicrobial-free or cycloheximide-treated cultures with high-CT substrate. Compared with low-CT substrate, BCVFA concentrations were reduced approximately 50% by high-CT substrate in antimicrobial-free and cycloheximide-treated cultures; BCVFA concentration was reduced by high-CT substrates by less than half that magnitude in cultures treated with penicillin + streptomycin. We interpreted this to suggest that fungal production of BCVFA partially compensated for that by bacteria when

bacterial fermentative activities were suppressed. Hassanat and Benchaar (2012) reported that BCVFA concentrations were not affected by the presence of CT at doses comparable with those used in our experiment; however, BCVFA concentrations increased, relative to tannin-free cultures, at CT doses less than those used in our study (i.e., 20 and 50 g CT/kg substrate).

In summary, CT had general suppressive effects on IVDMD, mean gas pressure, ammonia concentrations, total VFA concentrations, and individual VFA concentrations under the conditions of our study. This was likely due to tannin–protein interactions that decreased fermentative capacity of ruminal microbes. Suppression of bacterial fermentative capabilities markedly decreased total and individual VFA concentrations, whereas fungal suppression produced results similar to nonsuppressed cultures; therefore, we conclude that activities of ruminal fungi contributed little to major fermentation parameters during our 48 h in vitro incubations compared with those of bacteria. We further conclude that the influences of antimicrobial additives evaluated in our experiment produced similar effects on fermentation across inoculum-donor species.

Prior dietary exposure of ruminal fluid donors to CT mitigated the negative effects of dietary CT on total VFA and propionate concentrations; however, inoculum-donor species did not influence the adaptive response in our experiment. In spite of documented differences in CT sensitivity between beef cattle and small ruminants, we observed few noteworthy effects of inoculum-donor species. It is possible that such effects were overwhelmed by the influences of culture substrate, antimicrobial inclusion, and prior exposure to dietary CT under the conditions of our study. Alternatively, the extensively documented differences in CT tolerance between free-ranging herbivore species may be related to factors other than direct suppression of bacterial or fungal fermentative capabilities. Further research is warranted to characterize differences between beef cattle, sheep, and goats (roughage eaters, intermediate feeders, and concentrate selectors, respectively; Hofmann and Stewart, 1972) in eating behavior (e.g., meal size, meal duration, meal frequency, selectivity, and degree of mastication), ruminal digesta kinetics (e.g., rate of passage and extent of digestion), and endogenous CT-binding capabilities (e.g., parotid saliva; Lamy et al., 2010) and how those factors influence animal willingness to consume feedstuffs with elevated CT.

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