

EFFECTS OF CONDENSED TANNINS ON *IN VITRO* RUMINAL FERMENTATION

by

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A handwritten signature in black ink, appearing to read 'KC Olson', with a long horizontal flourish extending to the right.

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Abstract

Condensed tannins (CT) in plants are phenolic compounds with relatively high binding affinities for proteins. In ruminants, dietary CT limit DM intake and digestibility, and ruminal protein degradation by forming CT-protein complexes. Effects of dietary CT, animal species, prior dietary CT exposure, and antimicrobial inclusion on 48-h rate and extent of digestion were measured in two *in vitro* experiments. Cattle, sheep, and goats (n = 3 / species) were used in a 2-period, randomized complete-block experiment with a $2 \times 3 \times 2 \times 3$ factorial arrangement of treatments. Factor 1 was substrate: tannin-free or high-CT. Factor 2 was source of ruminal fluid inoculum: cattle, sheep, or goat. Factor 3 was prior animal exposure to a high-CT diet: non-exposed or exposed. Factor 4 was inclusion of antimicrobials: no antimicrobial, penicillin + streptomycin to suppress bacterial activity, or cycloheximide to suppress fungal activity in the fermentation. Tannin-free or high-CT substrates were incubated *in vitro* using ruminal fluid from animals either not exposed (period 1) or exposed to dietary CT (period 2). Periods consisted of an adaptation to tannin-free (10 d) or high-CT diets (21 d) and a 15-d period of ruminal-fluid collection via stomach tube. The presence of CT or penicillin + streptomycin in *in vitro* fermentation reduced ($P < 0.001$) total gas pressure, DM disappearance, and total VFA, acetate, propionate, butyrate, valerate, and branched-chain VFA concentrations. We concluded that: 1) CT had negative effects on fermentation, 2) prior exposure to dietary CT attenuated some but not all negative effects, and 3) CT effects were similar to the effects of penicillin + streptomycin.

Table of Contents

List of Figures	vi
List of Tables	viii
Acknowledgements	x
Chapter 1 - Review of Literature	1
Sericea Lespedeza	1
Seed Production	1
Canopy Dominance	2
Control	2
Tannins	3
Condensed Tannin Concentration	5
Protein-Precipitable Phenolics	5
Condensed Tannin Function	6
Dry Matter Intake	6
Diet Modifications: Corn Steep Liquor	7
Diet Modifications: Polyethylene Glycol	8
Ruminal Digestion Effects	9
Small Ruminants	12
Saliva	13
Anti-helminthic Effects	14
Conclusions	14
Literature Cited	15
Chapter 2 - Effects of High-Tannin Substrate, Prior Dietary Tannin Exposure, Antimicrobial Inclusion, and Animal Species on Mean Gas Pressure, Ammonia Concentration, Total VFA Concentration, and Individual VFA Concentrations Following a 48-h <i>In Vitro</i> Incubation	22
Abstract	22
Introduction	23
Materials and Methods	24
Study Preparation	24
Adaptation to Tannin-Free Diet	25

Adaptation to High-Tannin Diet	26
Ruminal Fluid Collection.....	27
Culture Substrate Preparation	29
48-h Batch Cultures	30
Laboratory Analyses	31
Statistical Analyses	32
Results and Discussion	32
Mean Gas Pressure.....	32
Ammonia Concentration.....	34
Total VFA Concentration	35
Acetate Concentration.....	36
Propionate Concentration.....	38
Butyrate Concentration	39
Valerate Concentration	39
Branched-Chain VFA Concentration.....	40
Literature Cited	42
Chapter 3 – Effects of High-Tannin Substrate, Prior Dietary Tannin Exposure, Antimicrobial Inclusion, and Animal Species on IVDMD, Ammonia Concentration, Total VFA Concentration, and Individual VFA Concentrations During a 48-h <i>In Vitro</i> Incubation	59
Abstract	59
Introduction.....	60
Materials and Methods.....	61
Study Preparation.....	61
Adaptation to Tannin-Free Diet	62
Adaptation to High-Tannin Diet.....	62
Ruminal Fluid Collection.....	64
Culture Substrate Preparation	65
<i>In Vitro</i> Rate of Digestion.....	66
Analytical Procedures	67
Statistical Analyses	72
Results and Discussion	72

<i>In Vitro</i> Dry Matter Disappearance	72
Ammonia Concentration	73
Total VFA Concentration	74
Acetate Concentration.....	76
Propionate Concentration.....	77
Butyrate Concentration	78
Valerate Concentration	79
Branched-Chain VFA Concentration.....	80
Literature Cited	84
Appendix A - 48-h Batch Culture Tables	102
Appendix B - <i>In Vitro</i> Rate of Digestion Tables	109

List of Figures

Figure 2.1 Effects of culture substrate and prior dietary tannin exposure on gas pressure following a 48-h <i>in vitro</i> incubation	45
Figure 2.2 Effects of culture substrate and antimicrobial on mean gas pressure following a 48-h <i>in vitro</i> incubation	46
Figure 2.3 Main effect of antimicrobial on ammonia concentration following a 48-h <i>in vitro</i> incubation.....	47
Figure 2.4 Effects of culture substrate and prior dietary tannin exposure on ammonia concentration following a 48-h <i>in vitro</i> incubation.....	48
Figure 2.5 Effects of culture substrate and prior dietary tannin exposure on total VFA concentration following a 48-h <i>in vitro</i> incubation.....	49
Figure 2.6 Effects of culture substrate and antimicrobial on total VFA concentration following a 48-h <i>in vitro</i> incubation	50
Figure 2.7 Effects of antimicrobial and prior dietary tannin exposure on total VFA concentration following a 48-h <i>in vitro</i> incubation.....	51
Figure 2.8 Effects of donor-species inoculum and culture substrate on acetate concentration following a 48-h <i>in vitro</i> incubation	52
Figure 2.9 Effects of culture substrate and prior dietary tannin exposure on acetate concentration following a 48-h <i>in vitro</i> incubation.....	53
Figure 2.10 Effects of culture substrate and antimicrobial on acetate concentration following a 48-h <i>in vitro</i> incubation.....	54
Figure 2.11 Effects of culture substrate, antimicrobial, and prior dietary tannin exposure on propionate concentration following a 48-h <i>in vitro</i> incubation	55
Figure 2.12 Effects of culture substrate and antimicrobial on butyrate concentration following a 48-h <i>in vitro</i> incubation	56
Figure 2.13 Effects of culture substrate and antimicrobial on valerate concentration following a 48-h <i>in vitro</i> incubation.....	57
Figure 2.14 Effects of culture substrate and antimicrobial on branched-chain VFA (isobutyrate + isovalerate) concentration following a 48-h <i>in vitro</i> incubation.....	58

Figure 3.1 Effects of fermentation time and culture substrate on <i>in vitro</i> dry matter disappearance during a 48-h incubation	87
Figure 3.2 Effects of culture substrate, donor-species inoculum, antimicrobial, and prior dietary tannin exposure on mean ammonia concentration during a 48-h <i>in vitro</i> incubation.....	88
Figure 3.3 Effects of fermentation time, culture substrate, and prior dietary tannin exposure on total VFA concentration during a 48-h <i>in vitro</i> incubation.....	89
Figure 3.4 Effects of fermentation time, culture substrate, and antimicrobial on total VFA concentration during a 48-h <i>in vitro</i> incubation.....	90
Figure 3.5 Effects of culture substrate and prior dietary tannin exposure on mean acetate concentration during a 48-h <i>in vitro</i> incubation.....	91
Figure 3.6 Effects of fermentation time, culture substrate, and antimicrobial on acetate concentration during a 48-h <i>in vitro</i> incubation.....	92
Figure 3.7 Effects of culture substrate and donor-species inoculum on mean propionate concentration during a 48-h <i>in vitro</i> incubation.....	93
Figure 3.8 Effects of fermentation time, culture substrate, and antimicrobial on propionate concentration during a 48-h <i>in vitro</i> incubation.....	94
Figure 3.9 Effects of donor-species inoculum and antimicrobial on mean butyrate concentration during a 48-h <i>in vitro</i> incubation	95
Figure 3.10 Effects of fermentation time, culture substrate, and antimicrobial on butyrate concentration during a 48-h <i>in vitro</i> incubation.....	96
Figure 3.11 Effects of donor-species inoculum and antimicrobial on mean valerate concentration during a 48-h <i>in vitro</i> incubation	97
Figure 3.12 Effects of fermentation time, culture substrate, and antimicrobial on valerate concentration during a 48-h <i>in vitro</i> incubation.....	98
Figure 3.13 Effects of culture substrate and donor-species inoculum on mean branched-chain VFA (isobutyrate + isovalerate) concentration during a 48-h <i>in vitro</i> incubation.....	99
Figure 3.14 Effects of fermentation time and donor-species inoculum on branched-chain VFA (isobutyrate + isovalerate) concentration during a 48-h <i>in vitro</i> incubation.....	100
Figure 3.15 Effects of fermentation time, culture substrate, and antimicrobial on branched-chain VFA (isobutyrate + isovalerate) concentration during a 48-h <i>in vitro</i> incubation.....	101

List of Tables

Table A.1 Effects of antimicrobial following a 48-h <i>in vitro</i> incubation	102
Table A.2 Effects of culture substrate and antimicrobial following a 48-h <i>in vitro</i> incubation ..	103
Table A.3 Effects of culture substrate and prior dietary tannin exposure following a 48-h <i>in vitro</i> incubation.....	104
Table A.4 Effects of donor-species inoculum and culture substrate following a 48-h <i>in vitro</i> incubation.....	105
Table A.5 Effects of antimicrobial and prior dietary tannin exposure following a 48-h <i>in vitro</i> incubation.....	106
Table A.6 Effects of culture substrate, antimicrobial, and prior dietary tannin exposure on total gas pressure and ammonia, total VFA, and acetate concentrations following a 48-h <i>in vitro</i> incubation.....	107
Table A.7 Effects of culture substrate, antimicrobial, and prior dietary tannin exposure on propionate, butyrate, valerate, and branched-chain VFA (isobutyrate + isovalerate) concentrations following a 48-h <i>in vitro</i> incubation	108
Table B.1 Effects of donor-species inoculum and antimicrobial during a 48-h <i>in vitro</i> incubation	109
Table B.2 Effects of donor-species inoculum and culture substrate during a 48-h <i>in vitro</i> incubation.....	110
Table B.3 Effects of culture substrate and prior dietary tannin exposure during a 48-h <i>in vitro</i> incubation.....	111
Table B.4 Effects of fermentation time and donor-species inoculum during a 48-h <i>in vitro</i> incubation.....	112
Table B.5 Effects of fermentation time, culture substrate, and antimicrobial on IVDMD and ammonia, total VFA, and acetate concentrations during a 48-h <i>in vitro</i> incubation	113
Table B.6 Effects of fermentation time, culture substrate, and antimicrobial on propionate, butyrate, valerate, and branched-chain VFA (isobutyrate + isovalerate) concentration during a 48-h <i>in vitro</i> incubation.....	114

Table B.7 Effects of donor-species inoculum, culture substrate, antimicrobial, and prior dietary tannin exposure on IVDMD and ammonia, total VFA, and acetate concentrations during a 48-h *in vitro* incubation115

Table B.8 Effects of donor-species inoculum, culture substrate, antimicrobial, and prior dietary tannin exposure on propionate, butyrate, valerate, and branched-chain VFA (isobutyrate + isovalerate) concentration during a 48-h *in vitro* incubation117

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Literature Review

Sericea Lespedeza

Sericea lespedeza (SL; *Lespedeza cuneata*) is a noxious, perennial weed that has invaded many parts of the Midwest, including the Flint Hills of Kansas (Eddy et al., 2003). *Sericea lespedeza* mainly grows in the eastern half of the United States, although it is adaptable to many soil types and climatic regimes (Gucker, 2010). The plant species was first imported to the United States from Japan in 1896 by the North Carolina Agriculture Research Station (Ohlenbusch et al., 2001). In 1924, the United States Department of Agriculture began propagating the plant to control erosion on roadsides and mine-reclamation sites. *Sericea lespedeza* was used also to control erosion in Kansas and Missouri after the Dust Bowl in the 1930s. During the 1940s, SL was planted as a pasture species for wildlife cover and as a forage crop due to its high protein content. The Conservation Reserve Program (CRP), part of the 1985 Farm Bill, encouraged the spread of SL by including the plant in native-seed mixtures to be planted on retired crop land (Ohlenbusch et al., 2007). *Sericea lespedeza* has rapidly spread in the United States over the past several decades; it is highly prolific due to drought tolerance, high seed production, allelopathic capabilities, dominant canopy, and high-tannin content (Eddy et al., 2003; Ohlenbusch et al., 2007; Vermeire et al., 2007). The State of Kansas established SL as a noxious weed in 2000. In 2003, an estimated 8.6 million acres of land in the US was infested by SL and another 5 million acres was listed at risk (Eddy, et al., 2003). From 1989 to 2003, SL infestation in Kansas increased from 25,000 to 500,000 acres (Duncan and Clark, 2005).

Seed Production

Individual SL plants can grow up to 201 cm in height with multiple stems (Bare, 1979; Stubbendiek and Conard, 1989). Branching occurs from the caudex and increases with age.

Sericea lespedeza has chasmogamous and cleistogamous flesses. Two different fless types allow the plants to utilize 3 different breeding systems: self-fertilization via cleistogamous flesses, self-fertilization via chasmogamous flesses, and insect pollination via chasmougamous flesses (Woods et al., 2008). Each SL stem can produce up to 1,500 seeds and many plants produce multiple stems (Ohlenbusch et al., 2007). Guernsey (1970) reported that one acre of cultivated SL could produce 150 to 300 million seeds. Seeds that were dormant in the soil for up to 20 years were still capable of germination; seed viability after that time was uncertain (Gucker, 2010). *Sericea lespedeza* had a fairly low germination rate without scarification (approximately 20-30%). With scarification, germination increased to 70 to 80% (Ohlenbusch et al., 2007; USDA, 2010).

Canopy Dominance

Sericea lespedeza preferred moist soil, but thrived in dry, infertile soils due to a large, branching taproot system (Ohlenbusch et al., 2007). When SL invaded an area, native grasses and forbs represented only 5% and 10% of canopy cover, respectively, compared to uninvaded areas where native grasses and forbs accounted for 79% and 28% of canopy cover, respectively (Eddy and Moore, 1998). Eddy et al. (2003) remarked that SL invasion was a severe threat to the native flora and fauna of the Flint Hills region and called for measures to decrease its spread.

Control

There are a number of options available for SL control but the effectiveness of each varies with location and degree of SL infestation. Herbicides decreased SL populations in one study at a cost of \$6.43 USD/ha (Silliman and Maccarone, 2005). Herbicides may not be effective or prudent to use on all landscapes because they are non-specific, killing desirable broad-leaf plants (Eddy et al., 2003).

Fall burning (on September 2) decreased stem height, stem number, and seedling density in surrounding areas by 32%, 75%, and 78%, respectively, relative to unburned control areas; moreover, the biomass of SL was reduced in the first growing season post-burning (Hamilton, 2003). Fire produced greater control of seedlings less than 6 wk old than seedlings 9 to 15 wk old. Patch burning, burning one-third of a pasture each year in a 3-year rotation, better controlled SL when compared to burning the entire pasture once per year; the yearly increase in percent cover decreased from 1.95% with annual burning to 0.47% with patch burning (Cummings et al., 2007). *Sericea lespedeza* invasion was diminished by minimizing soil disturbance, minimizing use of fertilizers containing nitrogen and phosphorus, cleaning equipment used in SL-infested areas, and regulating human and livestock contact with the affected sites until desired vegetation was established (Gucker, 2010).

Tannins

Biological control may be an effective option for preventing the spread of SL; moreover, grazing ruminants may be effective as an agent of biological control. The main issue with using grazing ruminant livestock as a means of biological control for SL are the high levels of tannins, an anti-nutritional compound (Eddy et al., 2003). Tannins play a vital role in plant defense mechanisms against diseases, stress, and herbivory. Tannins can limit DM intake, DM digestibility, and protein digestibility by ruminants (Makkar, 2003; Eckerle, 2011a).

Tannins are phenolic compounds found in a wide variety of plants and are most prevalent in legumes and browse species (Min et al., 2003). Phenolic compounds are classified as 2 specific groups: tannin phenolics which bind to proteins and non-tannin phenolics which do not bind to proteins. Tannin phenolics are further classified as either hydrolysable tannins (HT) or condensed tannins (CT). Both HT and CT are categorized as extractable or bound tannins

(Makkar, 2003). This review will be focused on CT because it is the main form of tannin found in SL and HT are less common in forages fed to or consumed by ruminants (Min et al., 2003).

Condensed tannins are polymers of flavanoid units connected by carbon-to-carbon bonds. Condensed tannins have a relatively high astringency, which is the measure of the capacity to bind to proteins and affect enzymatic activity (Waghorn, 2008). Condensed tannins bind to proteins and structural carbohydrates via hydrogen and hydrophobic bonding, rendering them unavailable to normal digestive and absorptive processes (Wroblewski et al., 2001). This interaction occurs due to the high affinity CT has for the carbonyl group of tertiary peptides (Haslam, 1989). Condensed tannins have variable molecular weights which can influence their affinity for proteins. Variations in the chemical structure of CT found among different plants can also affect the reactivity of CT.

Condensed tannins form complexes with proteins at neutral pH; at acidic pH the complex is disrupted and proteins can be digested by mammalian enzymes. The optimum pH at which proteins bind to CT is variable and depends on protein type and source. Most tannin-protein complexes form between pH 3.5 to 7 (i.e., across the range of normal ruminal pH). The complex dissociates at a pH less than 3.5 (normal abomasal pH; Min et al., 2003).

The binding affinity of CT is also influenced by chemical structure and molecular weight of proteins. These factors also affect the rate at which CT-protein complexes form. Condensed tannins have the strongest affinity for proteins containing relatively large concentrations of hydrophobic amino acids, proteins with open tertiary structures, and proteins with relatively large molecular weights. (Hagerman and Butler, 1991).

Condensed Tannin Concentration

According to Makkar (2003), the most common and accurate assay to determine CT concentration in plants is a colorimetric procedure known as the butanol-HCl-iron method. Condensed tannins, also referred to as proanthocyanidins, are comprised of polyhydroxyflavan-3-ol oligomers. Proanthocyanidins must be converted to anthocyanidins before CT concentration can be determined using the butanol-HCl-iron colorimetric assay. If samples are heat-treated, the proanthocyanidins may not completely be converted to anthocyanidins during the assay, resulting in an underestimation of CT. Temperatures above 50°C could inhibit the conversion. For this reason, samples should be freeze-dried instead of heat-dried for more accurate estimations. Not all CT in a sample are measureable because some occur in bound form, complexed to either proteins or carbohydrates. Purified CT may be obtained by extraction from a specific plant or via a commercially-available product. Commercial products are of a more consistent composition than laboratory extracts. Commercial products containing CT include spray-dried quebracho-extract tannins (QT), cyanidin, and delphinidin (Makkar, 2003).

Protein-Precipitable Phenolics

Protein precipitation methods were developed to more accurately determine the biological value of high-tannin feedstuffs. Protein-binding capacity was represented by the amount of tannins (phenolics) in a sample that precipitated protein. A complex was formed by adding bovine serum albumin (BSA) to a solution containing CT of feed origin. Then ferric-chloride reagent was added to the samples, which created a pink chromatophore when the iron reacted with the tannin-protein complex. UV-vis spectroscopy allowed for the absorbance of the sample to be determined and compared to a standard curve to estimate the amount of tannins present in the feed sample that bind to protein. Concentration of CT and their protein-binding

capacities in a given sample depended on the plant species, location of the plant, the climate in which the plant was grown, and growth stage of the plant (Makkar, 2003).

Condensed Tannin Function

Eckerle et al. (2010) determined that the peak in CT concentration of SL occurred in August, which corresponded to the flesching stage of the SL life cycle. The lowest CT concentration was in samples collected in June and October, which corresponded to the beginning and end of the growing season for SL, respectively. The protein-binding capacity of SL followed the same pattern as the CT concentration (Eckerle, 2010); SL had the greatest CT concentration and most potent protein-binding capacity during the middle of its growing season. Condensed tannins are used by plants as an anti-herbivory defense mechanism. Pathogen defense and energy and nitrogen conservation are also possible functions of CT in plants (Waghorn, 2008). Condensed tannins are toxic to some insects, deterring herbivory and reducing the number of insects that survive and reproduce (Forkner et al., 2004). The anti-herbivory defense works similarly in ruminants, but the effect is altered due to digestive differences between insects and ruminants. In ruminants, CT acts as an anti-nutrient rather than a toxin to reduce the DMI of forages (Waghorn, 2008).

Dry Matter Intake

Eckerle et al. (2011a, b, c) conducted a series of studies to determine the effects of SL on DMI of beef cattle. The first study was conducted to evaluate voluntary DMI of beef cows fed SL-contaminated hay or uncontaminated (SL-free) hay. All cows were fed uncontaminated tallgrass prairie hay for 20 d and voluntary DMI was monitored daily using a Calan-gate feeding system. Average DMI for all animals during this period was 113 ± 3.0 g/kg BW^{0.75}. On d 21, half of the cows were switched to SL-contaminated tallgrass prairie hay diet with similar CP%

and ADF% to the SL-free hay; DMI was monitored daily for 10 d. The SL-contaminated hay was 19.3% SL according to botanical composition estimates (200 to 250g CT/kg forage DM). The DMI for cows fed SL-contaminated hay was less (61 ± 8.9 g/kg $BW^{0.75}$) than the cows fed uncontaminated hay (112 ± 2.8 g/kg $BW^{0.75}$). The digestibilities of total-tract DM, CP, and NDF were monitored for the last 6 d of the study and were not different between cows fed uncontaminated and SL-contaminated hay diets. Total digestible DM was less for cows fed the SL-contaminated hay diet (29 ± 6.2 g/kg $BW^{0.75}$) than cows fed SL-free hay (64 ± 6.2 g/kg $BW^{0.75}$). This study demonstrated how SL suppressed the appetite of beef cattle under controlled conditions. They concluded that a biochemical interaction between CT and dietary proteins resulted in continuous decline of DMI over time.

Diet Modifications: Corn Steep Liquor

Corn steep liquor (CSL) is a byproduct of the wet corn milling. It is approximately 18% CP (DM basis) and is a suitable cattle feed due to low relative cost (Wagner et al., 1983). Eckerle et al. (2011b) evaluated how CSL affected voluntary DMI, total-tract digestibility, and total digestible DMI of beef cattle fed SL-contaminated hay. All animals were fed SL-contaminated tallgrass prairie hay *ab libitum* (19.3% SL; average DMI = 83 ± 2.2 g/kg $BW^{0.75}$) for 14 d before CSL supplementation began. Beef cows were randomly assigned to 1 of 4 CSL feeding levels: 0 (unsupplemented), 0.6, 1.2, or 1.8 kg DM/d offered once daily. Supplemented animals had greater DMI than unsupplemented animals. Animals fed 1.2 and 1.8 kg DM/d had greater total tract digestibility of CP and DM and greater total digestible DMI than unsupplemented animals and animals receiving 0.6 kg DM/d. This study demonstrated that CSL mitigated some of the negative effects of SL tannins on intake and digestibility in beef cattle.

In a subsequent study, Eckerle et al. (2011c) evaluated if supplementation with CSL could alleviate the effects of tannins to the point that beef cows would voluntarily consume a high-tannin diet (i.e. SL-contaminated hay). All animals had *ad libitum* access to uncontaminated (SL-free) and SL-contaminated tallgrass prairie hay simultaneously. The animals were randomly assigned to either unsupplemented (0 kg DM/d CSL) or supplemented (0.6 kg DM/d) treatment groups. Dry matter intake of the uncontaminated hay was not different between treatment groups. Dry matter intake of SL-contaminated hay by CSL-supplemented cows was approximately 25% greater than by unsupplemented cows. Additionally, total DMI and total-digestible DM were greater for CSL-supplemented cows than for unsupplemented cows. This study demonstrated that CSL could alleviate the negative effects of tannins enough to increase tolerance for and acceptance of a high-tannin diet by beef cows. Authors speculated that CSL-supplemented grazing animals may increase grazing pressure on SL and allow for a degree of biological control.

Diet Modifications: Polyethylene Glycol

Polyethylene glycol (PEG) was noted to be effective in binding to tannins and preventing them from forming complexes with proteins (Makkar, 2003). Mantz et al. (2009) conducted a trial to observe the effects of PEG supplementation on DMI of beef steers fed fresh-cut SL and prairie hay. The steers were randomly assigned to PEG-supplemented or unsupplemented treatment groups. The forage DMI of each animal was measured. The steers supplemented with PEG consumed more SL than unsupplemented steers. Preference tests indicated that steers supplemented with PEG selected more SL as a percentage of their diets than unsupplemented steers.

Ruminal Digestion Effects

According to Min et al. (2003), CT-protein complexes are first formed during mastication when CT are released from plant cells. Condensed tannins not bound to substrate can also bind to bacterial cells, but the effects of this interaction are not well understood. Free CT (i.e., that not associated with substrate or bacterial cells) may bind to extracellular enzymes and inhibit their effectiveness. Molan et al. (2000) determined that CT concentrations greater than 400 ug CT/mL of ruminal fluid would reduce the growth of several important bacterial strains. Hassanat and Benchaar (2012) reported that CT extracted from certain trees in the Anacardiaceae and Apocynaceae families, known commonly as quebracho trees, had dose-dependent effects on ruminal digestion parameters *in vitro*. Batch cultures were supplemented quebracho tannins (QT) at 0 (control), 20, 50, 100, or 200 g QT/kg substrate. Total VFA and NH₃ concentrations, butyrate and valerate molar proportions, and gas production of all QT-supplemented cultures were less than control cultures, whereas isobutyrate and isovalerate molar proportions were greater at 20 and 50 g QT/kg substrate doses than control cultures. At doses of QT \geq 100 g/kg, ruminal digestion parameters of QT-supplemented cultures that were lesser than controls included: gas production, total VFA concentration, butyrate molar proportion, valerate molar proportion, branched-chain VFA molar proportion, acetate: propionate ratio, methane production, and ammonia concentration. Ruminal digestion parameters that were greater than controls were final pH and propionate molar proportion. There were no differences in acetate molar proportions between control and QT-dosed cultures. These shifts in fermentation parameters demonstrated a possible negative effect of CT on fiber degradation by ruminal microorganisms. The reduction in total VFA concentration may have been due to the complexes that CT formed with carbohydrates and proteins (Makkar, 2003).

Tan et al. (2011) conducted a study to determine the effects of CT on ruminal fermentation parameters and methanogen populations *in vitro*. Batch cultures contained increasing amounts of CT: 0 (control), 10, 15, 20, 25, or 30 mg of CT mixed with 500 mg of dried guineagrass substrate. Ruminal fluid harvested from cattle was added to the cultures. Total gas production, methane production, CO₂ production, and *in vitro* DM degradability decreased with increasing amounts of CT; however, pH was not affected by the inclusion of CT. Total VFA concentrations were lesser in CT-treated cultures than in control cultures, but there were no differences between CT levels. Methane production also decreased with increasing CT inclusion. Reduction in methane production may have resulted from either reduced H⁺ availability (indicating a general decrease in OM fermentation) or from direct suppression of methanogens. Acetate and butyrate production by microbes resulted in excess H⁺ which was used by methanogens in the reduction of CO₂ to methane (Carulla et al., 2005; Tavendale et al., 2005; Patra and Saxena, 2010). Since gas production and total VFA production decreased, Hassanat and Benchaar (2012) concluded that reduction in OM digestion was the more likely cause of methane reduction.

Hervas et al. (2003) conducted *in situ* studies to determine the effect of CT on ruminal fermentation parameters in ewes. The ewes were ruminally-fistulated and assigned randomly to 1 of 4 QT treatments: 0 (control), 0.5, 1.5, and 3.0 g QT/kg BW daily. Treatments were administered intra-ruminally for 21 d. *In situ* studies were performed on d 0, 9, and 19 using barley straw as a substrate; CP and DM losses were determined. Disappearance of barley-straw DM, ADF, and NDF was less with 1.5 g QT/kg BW than with 0 or 0.5 g QT/kg BW/d on d 9 and d 19, demonstrating a dose-dependent decrease in fiber digestion with CT *in situ*. Animals given the largest QT dose (3.0 g/kg BW daily) were removed from the study on d 10 due to toxicity

issues and extremely poor ruminal fermentation. These ewes had almost complete inhibition of ruminal fermentation on d 9.

Getachew et al. (2008) evaluated the effects of QT on ruminal fermentation and microbial protein synthesis *in vitro*. Alfalfa hay was incubated in cattle ruminal fluid with increasing level of QT: 0 (control), 50, 100, or 150 g QT/kg alfalfa hay DM. Gas production was less in cultures with 100 and 150 g QT/kg DM compared to no QT. *In vitro* degradability of protein and ruminal NH₃-N concentrations decreased with increasing QT dose. *In vitro* digestibility of DM was less in cultures dosed with 100 g QT/kg DM than in control cultures and cultures dosed with 50 g QT/kg DM.

Ruminal protein degradation decreased with inclusion of CT *in vitro* (Bhatta et al., 2009; Pellikkaan et al., 2011; Hassanat and Bencharr, 2012) and *in vivo* (Carulla et al., 2005, Al-Dobaib, 2009). This may have been due to less protein digestion, fewer amino acids being deaminated, or less active ammonia-producing microbes. Min et al. (2005) conducted an *in vitro* study to determine the effects of CT on growth rate and microbial digestion of Rubisco (a major plant enzyme and protein) from white clover. The presence of CT reduced the degradation of Rubisco and growth rates in 11 bacterial strains common to the rumen compared to control (no CT). The degree of reduction was dependent upon bacterial strain and increased as inclusion of CT increased. They concluded that CT inclusion decreased some bacterial populations, decreased protein degradation by bacteria, and increased outflow of protein from the rumen. Min et al. (2002) also conducted an *in vivo* study with similar results. Sheep on a high-CT diet had decreased ruminal bacterial populations, nitrogen digestibility, and NH₃ concentrations. There was also an increase in outflow of feed nitrogen to the abomasum. These studies demonstrated a decrease in efficiency of protein degradation (Ulyatt et al., 1975).

Condensed tannins reduce the amount of OM that is digested by ruminal microbes, thus reducing total VFA concentration and total gas production. Condensed tannins also cause a reduction in MCP production efficiency and a reduction of total protein that is degraded or deaminated by proteolytic bacteria. Cumulatively, these effects result in decreased DMI, DM digestibility, and performance of ruminants. Waghorn (2008) compiled a list of the detrimental effects of tannins on digestion in ruminants: reduced ruminal protein digestion, reduced ruminal ammonia concentration, increased protein passage to the lower gastrointestinal tract, increased fecal nitrogen, reduced urinary nitrogen, reduced DM digestion, reduced voluntary DMI, increased ruminal volume, and reduced ruminal VFA production and concentrations. Tan et al. (2011) also demonstrated a decrease in methane production with CT, resulting in a reduced loss of gaseous energy; however, the decrease in methane production did not compensate for the major losses in digestive efficiency associated with CT consumption.

Small Ruminants

Small ruminants generally have greater tolerance for high-tannin forages than beef cattle. Frutos et al. (2004) conducted an *in vitro* study to evaluate the digestive differences between browsers (i.e., goats and deer) and grazers (i.e., cows and sheep). The fistulated animals in this study were adapted to an alfalfa-hay diet before collection of ruminal inoculum began. Batch cultures contained alfalfa hay (control; C) or C + QT. Gas production of cultures with goat and deer inoculum was not different between the C and C + QT treatments. Conversely, gas production was less in C + QT cultures with sheep and cow inoculum than C cultures with sheep and cow inoculum. *In vitro* DM disappearance was less in C + QT than C cultures for all species but the magnitude of decrease was less for goats and deer than for sheep and cows. The opposite was true of NH₃-N concentration. Ammonia-N was less in C + QT than C cultures for all species

and the magnitude of decrease was greater for goats and deer than for sheep and cows. Total VFA concentration was less in C + QT than C cultures for all species. This study demonstrated that ruminant species have different levels of tolerance for CT. Digestive parameters of ruminants that consume more browse than grass appear to be less effected by CT than ruminants that consume chiefly grass.

When SL was grazed heavily by goats, seed production was reduced from 950 to 3 seeds/ramet in 4 yr (Mayo, 2000). Pacheco et al. (2012) used a co-grazing system with beef cattle and goats to evaluate grazing pressure on SL. Native tallgrass pastures that were heavily infested with SL were randomly assigned to 1 of 2 treatments: cow-calf pairs only (single-species pastures) or cow-calf pairs and goats (multi-species pastures). Cow-calf pairs and goats were stocked at 0.8 AUM/ha. Cow body weights, calf average daily gain, and cow pregnancy rates were not different between treatment groups; however, cow body condition scores were greater in multi-species pastures than in single-species pastures. The amount of SL that was grazed in multi-species pastures was greater than that in single-species pastures (94.2% and 77.5%, respectively). Co-grazing may be a way to increase grazing pressure on SL, thereby reducing seed production; furthermore, co-grazing may allow beef producers to diversify and develop another stream of income.

Saliva

Saliva of certain ruminants may contain tannin-binding salivary proteins; these may serve as a primary defense against the negative effects of dietary CT. Tannin-binding salivary proteins are classified as either proline-rich or histidine-rich (Alonso-Diaz et al., 2012). Proline and histidine are relatively large, hydrophobic amino acids which bind strongly to CT during mastication. Goat saliva was reported to have elevated levels of proline and histidine compared

to sheep saliva. Goats fed a high-CT diet produced more parotid saliva when compared to goats fed a CT-free diet; moreover, the saliva produced by goats fed a high-CT diet had greater protein concentration than saliva produced by goats fed a CT-free diet (Lamy et al., 2010). Small ruminants had larger parotid salivary gland:BW than large ruminants (Vaithiyathan et al., 2001); increased production of saliva coupled with greater salivary protein levels may help to counteract negative effects of dietary CT and allow for greater tolerance of high-CT diets by small ruminants (Pacheco et al., 2012).

Anti-helminthic Effects

Negative digestion effects are caused when CT are consumed in moderate and high concentrations; however, CT can have beneficial effects for ruminants at low dietary concentrations. Condensed tannins were beneficial in preventing gastrointestinal nematode migration in sheep and goats *in vitro* and *in vivo* (Alonso-Diaz et al., 2008; Brunet et al., 2008; Martinez-Ortiz de Montellano et al., 2010). Min et al. (1999) found that ewes grazing high-CT forages had increased lambing percentages (25%) and wool production (14%) compared to animals grazing a CT-free pasture, possibly due to the anthelmintic effects of CT.

Conclusions

The ruminal fermentation effects of CT are generally detrimental to animal performance and overall digestion. Consumption of CT caused decreased DMI, ruminal protein and DM digestion, ruminal NH₃ concentrations, and ruminal VFA concentrations. Ruminal microbial activity was also reduced by the consumption of a high-CT diet. Small beneficial effects of low-level CT consumption have been reported; however, intake of CT is difficult to control under grazing conditions.

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Chapter 2 – Effects of High-Tannin Substrate, Prior Dietary Tannin Exposure, Antimicrobial Inclusion, and Animal Species on Mean Gas Pressure, Ammonia Concentration, Total VFA Concentration, and Individual VFA Concentrations Following a 48-h *In Vitro* Incubation

Abstract

Effects of dietary condensed tannins (CT), prior dietary CT exposure, animal species, and antimicrobial inclusion on 48-h extent of *in vitro* fermentation were measured. Cows, sheep, and goats (n = 3 / species) were used in a 2-period, randomized complete-block experiment with a 2 × 3 × 2 × 3 factorial arrangement of treatments. Factor 1 was culture substrate: high-CT or tannin-free. Factor 2 was inoculum-donor species: *Bos taurus*, *Ovis aries*, or *Capra hircus*. Factor 3 was prior donor-species exposure to a high-CT diet: non- exposed or exposed. Factor 4 was antimicrobial: no antimicrobial, bacterial suppression (penicillin + streptomycin), or fungal suppression (cycloheximide). Tannin-free or high-CT substrates were incubated *in vitro* using ruminal fluid from animals either not exposed to dietary CT (period 1) or previously exposed to dietary CT (period 2). Periods consisted of an adaptation to tannin-free (10 d) or high-CT diets (21 d) and a 15-d period of ruminal-fluid collection via stomach tube. Cultures fed high-CT substrate or that were spiked with penicillin + streptomycin had less (P < 0.001) gas production, and less (P < 0.001) total VFA, acetate, propionate, butyrate, valerate, and branched-chain VFA concentrations than cultures fed tannin-free substrate and cultures that were either antimicrobial-free or spiked with cycloheximide. In tannin-free cultures, total VFA concentration was greater (P < 0.001; LSD = 3.43) when ruminal fluid was from animals without prior CT exposure (83.7 mM) than when ruminal fluid was from tannin- exposed animals (79.6 mM). Conversely, in

high-CT cultures, total VFA concentration was greater with tannin- exposed ruminal fluid (59.4 mM) than with non- exposed ruminal fluid (52.6 mM). In tannin-free cultures, acetate concentrations were greater (LSD = 5.89) in cultures with no donor-animal exposure to CT than in cultures with inoculum from CT- exposed donors (57.8 and 51.3 mM). Cultures with high-CT substrate had the least acetate concentrations; however, there was no difference among them with respect to tannin- exposure status of donor animals (36.7 and 37.3 mM for non- exposed and exposed, respectively). In cultures with CT-exposed ruminal fluid, propionate concentration was greater (LSD = 3.77) in antimicrobial-free cultures fed tannin-free substrate (27.2 mM) than in antimicrobial-free and cycloheximide-spiked cultures fed high-CT substrates (21.6 and 22.1 mM, respectively). We concluded that: 1) CT had general deleterious effects on fermentation and 2) prior exposure to dietary CT attenuated some negative effects of CT consumption on fermentation during a 48-h batch-culture *in vitro* incubation.

Key words: condensed tannins, dietary tannin adaptation, *in vitro* fermentation

Introduction

Condensed tannins (CT) are polymers of flavanoid units connected by carbon-to-carbon bonds; they have a relatively high capacity to bind to proteins and affect enzymatic activity (Waghorn, 2008). Condensed tannins are found in a wide variety of plants and are most prevalent in legume and browse species; CT are a functional defense mechanism against diseases, stress, and herbivory (Min et al., 2003). Tannins limit DM intake, DM digestibility, and ruminal protein degradation by ruminants due to formation of CT-protein complexes *in vivo* (Makkar, 2003; Eckerle, 2011). Condensed tannin-protein complexes are formed during mastication when CT are released from plant cells (Min et al., 2003). These complexes are maintained under ruminal conditions and render proteins unavailable to ruminal microbes

(Makkar, 2003). Ruminal protein degradation decreased with the addition of CT *in vitro* (Hassanat and Bencharr, 2012) and *in vivo* (Al-Dobaib, 2009). Hassant and Benchaar (2012) demonstrated a decrease in total VFA concentration and 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 select species of ruminal bacteria. Small ruminants reportedly had 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 tannins in *Bos taurus*, *Ovis aries*, and *Capra hircus*, with and without selective antimicrobial suppression of either ruminal bacteria or ruminal fungi on mean gas pressure, ruminal ammonia concentrations, and ruminal concentrations of total and individual VFA in 48-h *in vitro* batch cultures.

Materials and Methods

Study Preparation

The Kansas State University Institutional Animal Care and Use Committee reviewed and approved all animal-handling and animal-care practices used in our research (protocol no. 3423). Three beef cows (*Bos taurus*; 551 ± 30 kg BW), 3 sheep (*Ovis aries*; 68 ± 3 kg BW), and 3 goats (*Capra hircus*; 49 ± 4 kg BW) were used in this experiment. Sheep and goats were housed together in a 10 x 10 m pen and cows were housed in an adjacent 100 x 100 m pen. Smooth brome grass hay (*Bromus inermis*; 87.9% DM; 9.1% CP, 76.2% NDF, and 47.0% ADF) was offered to all animals daily in round-bale feeders (diameter = 2.5 m) in amounts calculated to

allow *ad libitum* intake ($\approx 4\%$ BW/d). One animal from each species was assigned randomly to 1 of 3 cohorts; cohorts were assigned randomly to 1 of 3 sampling times during each of 2 experimental periods. Animals were fed a single tannin-free diet during period 1 and a single high-CT diet during period 2. Tannin-free and tannin-contaminated substrates were subject to *in vitro* fermentation using ruminal inoculum harvested from *Bos taurus*, *Ovis aries*, and *Capra hircus* that were either not exposed to dietary CT (period 1) or exposed to dietary CT (period 2).

Adaptation to Tannin-Free Diet

The timeline of our experiment was expressed relative to the first day of animal adaptation (d 1) to treatment diets. All animal cohorts were fed tannin-free smooth brome grass hay *ad libitum* for 10 d to begin period 1. Ruminal fluid was collected via stomach tube from animals from d 11 to 17 for use in *in vitro* batch cultures. Ruminal fluid was collected from cohort 1 on d 11, from cohort 2 on d 14, and from cohort 3 on d 17. During the period of ruminal fluid collection, animals continued to be fed for *ad libitum* intake of smooth brome grass hay, as during the adaptation phase of the experiment. All animals had continual, unrestricted access to fresh water, a salt block (98.0% NaCl; Compass Minerals, Chicago, IL) and a mineral block (95.5% NaCl, 3500 ppm Zn, 2000 ppm Fe, 1800 ppm Mn, 280 ppm Cu, 100 ppm I, and 60 ppm Co; Compass Mineral, Chicago, IL) during period 1. The tannin-free diet was assumed to allow no exposure to CT.

Adaptation to High-Tannin Diet

During period 2, animal subjects were adapted to high-CT intake conditions by providing them with grain-byproduct supplements that were spiked with quebracho tannins. Hassanat and Benchaar (2013) reported that condensed tannins extracted from certain trees in the *Anacardiaceae* and *Apocynaceae* families, known commonly as quebracho trees, had dose-

dependent effects on ruminal digestion parameters *in vitro*. Purified, feed-grade quebracho-tannin extract (QT) was procured from Wintersun Chemical (Ontario, CA) for use in high-CT supplements and high-CT culture substrates.

Immediately following period 1, animal subjects were fed tannin-free smooth bromegrass hay *ad libitum* for an additional 21 d. Each animal was also individually fed a supplement which contained QT at 0.1% BW/d, soybean hulls at 0.2% BW/d, and dried molasses at 0.05% BW/d. Soybean hulls and molasses were fed to encourage complete consumption of the prescribed dose of QT. The animals were individually penned each morning (0730) and each evening (2000) 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 DMI. Supplement consumption by sheep and goats was complete each day, whereas cows left an average of 183 ± 10 g QT unconsumed daily. Consumed QT was equivalent to only 67% of the targeted dose, underscoring the strong aversion among beef cows to dietary CT reported by Eckerle et al. (2011). Animals were fed the tannin-containing supplement in conjunction with *ad libitum* smooth bromegrass hay for 21 d before ruminal-fluid collections began. All animal subjects had continual access to fresh water, a salt block, and a mineral block as in period 1.

Ruminal fluid was collected via stomach tube from d 22 to 28 from animals for *in vitro* batch cultures. Ruminal fluid was collected from cohort 1 on d 22, from cohort 2 on d 25, and from cohort 3 on d 28. During the period of ruminal fluid collection, animals continued to be fed for *ad libitum* intake of smooth bromegrass hay, as during the adaptation phase of the experiment; moreover, they continued to be fed QT-containing supplements daily.

Adaptation to the high-CT diet was considered to provide prior exposure to CT. Although our measurements of the effects of tannin-free and high-CT diets were separated in time, we assumed that the time difference did not confound our comparisons. Animal subjects, facilities, and the conditions under which *in vitro* techniques were used during periods 1 and 2 were held constant.

Ruminal Fluid Collection

Ruminal fluid was collected orally at 0730 on the schedule designated for each animal cohort. A simple vacuum strainer was used for this purpose. The vacuum strainer was constructed from a 2-L sidearm flask fitted with a #6 rubber stopper pre-drilled with two 1-cm holes. A 50 x 1 cm polyethylene tube was connected to the stopper via a 1-cm OD fitting that penetrated the stopper approximately 2 cm on each side. The distal end of the tube was connected to a 500-mL vacuum bottle. The vacuum bottle, in turn, was connected to an electrical vacuum pump. The purpose of the intervening vacuum bottle was to prevent fluids from inadvertently being drawn into the pump.

Another polyethylene tube (300 x 1 cm) was placed in the second hole in the stopper, penetrating it by approximately 10 cm. A copper cylinder (10 x 1 cm) was fitted to the distal end of the tube. The copper cylinder was prepared for use as a coarse-particle filter by drilling 0.5-cm holes at approximately 0.25-cm intervals along its length and around its circumference. When the vacuum pump was engaged, the pump operator covered the sidearm opening in the flask with a finger to create suction manually. Manual control of suction allowed for the collection device to be adjusted or cleaned rapidly during ruminal-fluid collection.

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 biting the collection tube, thereby maintaining fluid flow or preventing damage to the tube. The collection tube, detached from the sidearm flask, was inserted copper-fitting side first into the esophagus. Once the tube reached the cardia, air was blown into it to force the cardia open and allow gentle passage of the tube into the rumen. The collection tube was twisted slightly to force the brass fitting below the fiber mat. The tube was then attached to the sidearm flask. The pump was turned on and fluid collection began.

To prevent excessive salivary contamination, the first 200 mL of ruminal fluid collected from each animal was discarded. Several times during each collection, the collection tube was detached from the sidearm flask and air was blown into the tube clear digesta from the copper fitting. Approximately 700 mL of fluid were harvested from each animal at each collection; collection time was approximately 7 min / animal. The collection tube was pulled slowly out of the esophagus upon completion and the speculum was removed. Harvested ruminal fluid from each animal was transferred immediately to a single 3-L, pre-warmed, insulated bottle for transport to laboratory facilities. Harvested ruminal fluid was strained through 8 layers of cheesecloth and poured into a separatory flask; it was allowed to separate in the flask for 30 min. The heaviest fraction, containing protozoa and other waste, was discarded.

Culture Substrate Preparation

During periods 1 and 2, the tannin-free substrate added to ruminal inoculum of cattle, sheep, and goats was smooth bromegrass hay (87.9% DM; 9.1% CP, 76.2% NDF, and 47.0% ADF). Hay was dried at 55°C for 48 h and ground (#4 Wiley Mill, Thomas Scientific, Swedesboro, NJ, USA) to pass a 1-mm screen before it was added to culturing devices. The tannin-contaminated substrate added to ruminal inoculum of cattle, sheep, and goats during

periods 1 and 2 was a composite of the smooth bromegrass hay used as the tannin-free substrate and CT in the form of QT. Hay and QT were blended to achieve a CT concentration of 10.2% (wt/wt; Eckerle et al., 2010). 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, 0.5% NDF, and 2.7% ADF.

In order to determine the relative importance of bacterial and fungal fermentative activities *in vitro*, tannin-free and tannin-contaminated substrates were subject to *in vitro* fermentation with 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., Milwaukee, WI). A bacterial-suppression solution was prepared containing 12.5 mg of penicillin G and 2 mg of streptomycin sulfate per mL of deionized water. One mL of this solution was added to *in vitro* cultures per 10 mL of ruminal fluid. Fungal suppression was accomplished by preparing a solution of 5 mg of cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) per mL of deionized water. One mL of fungal-suppression solution was added to *in vitro* cultures per 10 mL of ruminal fluid. Unsuppressed *in vitro* cultures were examined concurrently for reference.

48-h Batch Cultures

In vitro total gas production, ammonia concentrations, total VFA concentrations, and individual VFA concentrations were measured using a 48-h batch-culture technique. Cultures were conducted using 250-mL glass jars equipped with ANKOM gas-production system lids (Model RF1; Ankom Technology, Macedon, NY). Nine jars were used to measure effects of treatment for each animal in each cohort in a $2 \times 3 \times 2 \times 3$ factorial arrangement of a randomized

complete block design. Factor 1 was culture substrate: high-CT or tannin-free. Factor 2 was donor-animal species: *Bos taurus*, *Ovis aries*, or *Capra hircus*. Factor 3 was prior exposure to CT: non-exposed or exposed. Factor 4 was antimicrobial: no antimicrobial, bacterial suppression (penicillin + streptomycin), or fungal suppression (cycloheximide).

Within animal and cohort, 6 jars were assigned randomly to receive substrate, ruminal fluid, and McDougal's artificial saliva and 3 were assigned randomly to serve as blanks, receiving ruminal fluid and McDougal's artificial saliva only. McDougal's artificial saliva was prepared 24 h before each ruminal-fluid collection and stored in an incubator at 39°C. Ruminal fluid and McDougal's artificial saliva were included in the cultures at a 1:2 ratio. Blank jars were randomly assigned to receive no additive, penicillin + streptomycin sulfate, or cycloheximide. Jars containing substrate were randomly assigned to receive tannin-free substrate (3 g smooth brome grass hay) or high-CT substrate (2.87 g smooth brome grass hay + 0.48 g QT); within each substrate group (n = 3), jars were randomly assigned to receive no additive, penicillin + streptomycin sulfate, or cycloheximide.

Five mL of penicillin + streptomycin sulfate solution were added to designated samples and 5 mL of cycloheximide solution were added to designated samples immediately before ruminal fluid was added to culture jars (Windham and Akin, 1984). Fifty mL of ruminal fluid from each species were added to jars, including blanks, followed by 100 mL McDougal's artificial saliva. Jars were individually gassed with N₂ for 20 s and capped with an ANKOM RF1 lid. The jars were placed in an incubator (Model G25; New Brunswick Scientific Co., Inc., New Brunswick, N.J.) in random order at 39°C for 48 h. After 48 h, jars were removed and immediately placed on ice to cease fermentation.

Gas pressure in the headspace of each jar was recorded every 15 min during the 48-h incubation. 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 meta-phosphoric acid (25%, w/v) had been added to suspend microbial activity. Samples were frozen (-20°C) pending analysis.

Laboratory Analyses

Sample concentrations of total and individual VFA were measured via gas chromatography. Before VFA analyses, samples were thawed for 30 min, agitated for 10 s with a vortex mixer, and centrifuged at $16,000 \times g$ for 10 min (Eppendorf model 5415C; Brinkmann Instruments, Inc., Wesbury, NY). The supernatant from each sample was transferred to a 2-mL gas-chromatography vial. A 1.8 m x 6 mm, 4-mm i.d. glass column packed with GP 10% SP-1200 / 1% H₃PO₄ (Supelco #1-1965; Sigma-Aldrich) was used for analyses. Nitrogen was the carrier gas (flow rate = 80 mL/min); detection was by flame ionization (compressed air flow = 200 mL/min, H₂ flow = 20 mL/min, combined flow = 200 mL/min). The injection and detector were set at 250°C and the column temperature was maintained between 120 and 140°C.

Ammonia concentrations were analyzed via uv/vis spectroscopy using a Technicon Autoanalyzer II (Technicon Industrial Systems, Tarrytown, NY) according to procedures described by Broderick and Kang (1980). 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., Macedon, NY), and ADF (AOAC, 2005; 973.18 modified for the Ankom 200 fiber analyzer, Ankom Technology Corp.).

Statistical Analyses

Gas pressure, concentrations of ammonia, and concentrations of total VFA, acetate, propionate, butyrate, valerate, and branched-chain VFA (i.e. isobutyrate + isovalerate) concentrations were analyzed using a $2 \times 3 \times 2 \times 3$ factorial arrangement of a randomized complete block design (PROC MIXED; SAS Inst. Inc., Cary, NC). Class factors included animal species (*Bos taurus*, *Ovis aries*, or *Capra hircus*), culture substrate (tannin-free or high-CT), microbial suppressant (none, penicillin + streptomycin, or cycloheximide), animal cohort (i.e., block; 1, 2, or 3), and animal exposure to tannins (non-exposed or exposed). The model statement included terms for the fixed effects of animal species, culture substrate, microbial suppressant, dietary tannin exposure, and all possible 2-, 3-, and 4-way interaction terms. The random statement had terms for animal cohort, cohort \times species, and dietary tannin exposure (cohort \times species). When protected by a significant F-test ($P < 0.001$), means were separated using the method of Least Significant Difference. Least-squares means for the highest-order, significant ($P < 0.001$) interaction term were reported.

Results and Discussion

Gas Pressure

Effects of culture substrate on gas pressure were influenced ($P = 0.0002$; culture substrate \times tannin-exposure status) by donor-animal exposure to dietary tannins. Mean gas pressure was greater (LSD = 0.21) in cultures fed tannin-free substrate (1.83 and 1.65 bar for non-exposed and exposed, respectively) than for cultures fed high-CT substrate (0.82 and 0.94 bar for non-exposed and exposed, respectively; Figure 2.1). Tannin-exposure status of donor inoculum did not affect gas pressure averaged over substrate type. 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 condensed tannins. In our study, prior exposure to dietary tannins did not alleviate depression in gas pressure *in vitro*.

Effects of culture substrate on mean gas pressures were also *influenced* ($P < 0.0001$) by antibiotic addition to 48-h *in vitro* batch cultures (Table 2). Mean gas pressures were not different ($LSD = 0.24$) between antibiotic-free cultures fed tannin-free substrate and cycloheximide-spiked cultures fed tannin-free substrate (2.11 and 1.98 bar, respectively). In addition, mean gas pressures were not different between antibiotic-free cultures fed high-CT substrate and cycloheximide-spiked cultures fed high-CT substrate (0.99 and 1.02 bar, respectively). We interpreted these data to indicate that fermentative activities of ruminal fungi contributed little to total gas production in a 48-h batch-culture *in vitro* system. Mean gas pressures in tannin-free cultures that were spiked with penicillin + streptomycin (1.14 bar) were less than in tannin-free cultures without antibiotic and tannin-free cultures spiked with cycloheximide. High-CT substrate also produced lesser gas pressures when dosed with penicillin + streptomycin (0.65 bar) than when no antibiotic was added to high-CT cultures or when high-CT cultures were dosed with cycloheximide. Clearly, suppression of bacterial fermentative activities had strong negative effects on production of gas in a 48-h, batch-culture *in vitro* system. Windham and Akin (1984) indicated that the ruminal bacteria were responsible collectively for more fiber degradation than the ruminal fungi; therefore, bacteria may have contributed more to total gas production from a fibrous substrate in our study than fungi.

Ammonia Concentration

Antimicrobial treatment response was not influenced ($P \geq 0.13$) by culture substrate type, by donor animal species, by prior donor-animal exposure to dietary tannins, or by any interactions between these factors; therefore, main effects of antimicrobial treatment on ammonia concentration were reported (Figure 2.3). Ammonia concentration was greatest ($P < 0.0001$; LSD = 1.19) in cultures spiked with penicillin + streptomycin, slightly less in cultures not treated with antimicrobial, and least in cultures spiked with cycloheximide (20.2, 19.0, and 16.8 mM, respectively). Satter and Slyter (1974) indicated that ammonia concentration in an *in vitro* system was influenced by both amino-acid deamination and amino-acid synthesis by ruminal microbes. We speculated the balance between these activities was affected differentially by suppression of fungal or bacterial fermentative activities relative to the antimicrobial-free control. In our study, fungal suppression may have led to greater net ammonia uptake by bacteria, whereas bacterial suppression may have decreased ammonia uptake.

Ammonia concentration was influenced ($P < 0.0001$; culture substrate \times tannin-exposure status) by both culture substrate and tannin-exposure status of ruminal fluid donors (Figure 2.4). Concentration of ammonia was greatest (LSD = 2.05) in cultures fed tannin-free substrate (19.4 and 19.9 mM for non-exposed and exposed, respectively), regardless of tannin-exposure status, and least in cultures that were fed high-CT substrate and inoculated with ruminal fluid from tannin-exposed animals (16.7 mM); ammonia concentration in cultures with non-tannin exposed ruminal inoculum and fed a high-CT substrate was intermediate (18.5 mM). Ruminal ammonia concentration was reduced in the presence of dietary tannins due to decreased ruminal protein degradation (Frutos et al., 2004; Hassanat and Benchaar, 2012). We speculated that prior dietary exposure to tannins may have reduced populations of microbial species that depended on free ammonia as a N source.

Total VFA Concentration

Total VFA concentrations in 48-h *in vitro* batch cultures were influenced ($P < 0.001$; culture substrate \times tannin exposure status) by culture substrate and tannin-exposure status of ruminal fluid donors (Figure 2.5). Total VFA concentration was greatest (LSD = 3.43) when tannin-free medium was fed to cultures inoculated with ruminal fluid from animals without prior exposure to dietary CT (83.7 mM); it was slightly less in cultures fed tannin-free substrate and incubated with tannin-exposed inoculum (79.6 mM). In contrast, total VFA concentration decreased sharply in cultures fed high-CT substrate; moreover, cultures inoculated with tannin-exposed ruminal fluid had greater total VFA concentration than cultures inoculated with non-exposed ruminal fluid (59.4 and 52.6 mM, respectively). Culture substrates containing tannin generally depressed total VFA concentration compared with cultures fed tannin-free substrate. Prior exposure to dietary CT ameliorated this depression. Condensed tannins were noted to depress total VFA concentration (Waghorn, 2008; Tan et al., 2011; Hassanat and Benchaar, 2012). We speculated that prior dietary exposure to condensed tannins may have alleviated a portion of their detrimental effects on total VFA concentration in ruminants.

Effects of culture substrate on total VFA concentration were influenced ($P < 0.001$; culture substrate \times antimicrobial) by antimicrobial inclusion in cultures (Figure 2.6). Total VFA concentration was greatest (LSD = 3.43) in cultures fed tannin-free media without antimicrobial additives; they were slightly less for cultures fed tannin-free media and dosed with cycloheximide (96.1 and 91.6 mM, respectively). Both produced significantly greater VFA concentration at 48 h of incubation than antimicrobial-free, high-CT substrate cultures and cycloheximide-treated, high-CT cultures (63.6 and 62.7, mM, respectively). Cultures treated with penicillin + streptomycin had lesser total VFA concentration than other culture types. Within cultures treated with penicillin + streptomycin, tannin-free substrate produced more total VFA

than tannin-contaminated substrate (57.2 and 41.7 mM, respectively). Molan et al. (2000) determined that CT concentrations > 400 ug CT / mL of ruminal fluid reduced growth of several important bacterial strains. This may have exacerbated the effects of the high-CT substrate and penicillin + streptomycin treatment in our study.

Effects of antimicrobial treatment on total VFA concentration were influenced ($P = 0.001$; antimicrobial \times tannin-exposure status) by tannin-exposure status of ruminal fluid donors. Within antimicrobial-free cultures, total VFA concentrations were not different ($LSD = 8.51$) between sources of donor inoculum that were not exposed or exposed to dietary CT (78.2 and 81.5 mM, respectively; Figure 2.7). Total VFA concentration was also not different between non-exposed and CT-exposed inoculum for penicillin + streptomycin-spiked cultures (50.8 and 48.0 mM, respectively) or cycloheximide cultures (75.4 and 78.9 mM, respectively). Total VFA concentration was not different between antimicrobial-free cultures and cycloheximide-treated cultures; however, both had greater total VFA concentration than cultures treated with penicillin + streptomycin. Suppression of bacterial fermentative activities had a strong negative influence on total VFA concentration, whereas suppression of fungal fermentative activities had no influence on total VFA concentration, relative to cultures not treated with antimicrobial.

Acetate Concentration

An interaction between culture substrate and donor-species inoculum was detected ($P < 0.001$) for acetate concentration. *Bos taurus* and *Capra hircus* inoculum with tannin-free substrates had the greatest ($LSD = 2.96$) acetate concentrations after 48 h of incubation; *Ovis aries* inoculum with tannin-free substrate had less acetate (57.1, 55.0, and 51.6 mM, respectively; Figure 2.8). Cultures with high-CT substrate had less acetate than cultures with tannin-free substrate across all species. Interestingly, high-CT substrates depressed acetate

concentrations approximately 30% across donor species compared with tannin-free substrates. Tan et al. (2011) and Hassanat and Benchaar (2012) reported no change in acetate molar proportion in the presence of condensed tannins. We speculated that CT depressed fiber degradation resulting in lesser acetate concentration. We concluded that donor-animal species had little influence on acetate concentration following a 48-h *in vitro* incubation under the conditions of our study.

A second interaction for acetate concentration was detected between culture substrate and CT-exposure status of donor animals ($P < 0.001$). In tannin-free cultures, acetate concentrations were greatest (LSD = 5.89) in cultures with no donor-animal exposure to tannins and less in cultures with inoculum from CT-exposed donors (57.8 and 51.3 mM, respectively; Figure 2.9). Apparently, prior tannin exposure may have depressed acetate yield, even when CT were not present in the diet. Cultures with high-CT substrate had the least acetate concentrations; however, there was no difference among them with respect to CT-exposure status of donor animals (36.7 and 37.3 mM for non-exposed and exposed, respectively). Under the circumstances of our study, prior tannin exposure may have spared acetate yield.

Acetate concentrations were also influenced ($P < 0.001$) by a culture substrate \times antimicrobial treatment interaction (Figure 2.10). In tannin-free cultures, acetate concentrations were modestly increased (LSD = 2.96) in cultures without antimicrobial compared with cultures dosed with cycloheximide; both had greater acetate concentrations than cultures dosed with penicillin + streptomycin (60.5, 57.5, and 45.6 mM, respectively). Cultures fed tannin-free substrate had greater acetate concentrations than cultures fed high-CT substrate. Among cultures fed high-CT substrates, those with no antimicrobial 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).

Propionate Concentration

A three-way interaction effect was detected ($P < 0.001$) for propionate concentration following a 48-h *in vitro* incubation (Figure 2.11). Propionate concentration was greater (LSD = 3.77) in antimicrobial-free cultures fed tannin-free substrate and inoculated with tannin-exposed ruminal fluid than in antimicrobial-free and cycloheximide-spiked cultures fed high-CT substrates with prior CT exposure; propionate concentrations were intermediate in non-CT exposed, antimicrobial-free cultures and in cycloheximide-treated cultures fed tannin-free media, as well as in tannin-exposed, cycloheximide-treated cultures fed tannin-free media. Each of the aforementioned treatments had greater propionate concentrations than non-CT exposed cultures fed high-CT substrate and either not treated with an antimicrobial compound or treated with cycloheximide. In contrast, treatment with penicillin + streptomycin produced the least concentrations of propionate; substrate type and tannin exposure had no effect on propionate yield when bacterial activities were suppressed. Tan et al. (2011) reported no change and Hassanat and Benchaar (2012) reported a slight increase in propionate molar proportions in the presence of CT compared to controls. We generalized that propionate-producing organisms in our study, 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.

Butyrate Concentration

Effects of culture substrate on butyrate concentration were influenced ($P < 0.001$) by antimicrobial treatment (Figure 2.12). Within each substrate type (i.e., tannin-free or high-CT),

cultures not treated with an antimicrobial additive had the greatest (LSD = 0.389) butyrate concentrations (7.1 and 4.4 mM for tannin-free and high-CT cultures, respectively), cycloheximide-spiked cultures were intermediate (6.4 and 4.0 mM for tannin-free and high-CT cultures, respectively), and cultures spiked with penicillin + streptomycin had the least butyrate concentrations (3.6 and 2.9 mM for tannin-free and high-CT cultures, respectively). Across antimicrobial treatments, cultures fed high-CT substrate had lesser concentrations of butyrate than cultures fed tannin-free substrates. Relative to antimicrobial-free cultures, butyrate concentrations were slightly reduced (< 1 mM) by anti-fungal treatment and extensively reduced (1.5 to 3.5 mM) by anti-bacterial treatment. We interpreted 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).

Valerate Concentration

Valerate concentrations were influenced ($P < 0.001$) by culture substrate and antimicrobial treatment (Figure 2.13). Within each substrate type, valerate concentrations were not different (LSD = 0.160) between antimicrobial-free cultures and cultures treated with cycloheximide; however, tannin-free substrate was associated with greater valerate concentration than high-CT substrate for these treatments (0.8 vs. 0.4 mM for antimicrobial-free cultures and 0.7 vs. 0.4 mM for cycloheximide-spiked cultures). Valerate concentration was least in cultures treated with penicillin + streptomycin (0.2 mM for both tannin-free and high-CT cultures). Substrate type did not influence valerate concentration within penicillin + streptomycin-treated cultures. Similar responses to antimicrobial treatment and substrate type were noted with total

VFA concentration following a 48-h *in vitro* incubation. Reduction in valerate molar proportion in the presence high-CT substrate was reported also by Getachew et al. (2008) and Hassanat and Benchaar (2012). We concluded the ruminal fungal population did not significantly contribute to valerate concentration under the conditions of our study.

Branched-Chain VFA Concentration

Concentrations of branched-chain VFA (isobutyrate + isovalerate; BCVFA) were influenced ($P < 0.001$) by culture substrate and antimicrobial treatment (Figure 2.14). Within cultures fed tannin-free substrate, antimicrobial-free cultures had the greatest (LSD = 0.148) BCVFA concentrations, cultures treated with cycloheximide were intermediate, and cultures treated with penicillin + streptomycin had the least BCVFA concentrations (1.6, 1.2, and 1.0 mM, respectively). Cultures fed high-CT substrate had lesser BCVFA than cultures fed tannin-free substrate. Antimicrobial-free cultures fed high-CT substrate had greater BCVFA than cycloheximide-treated cultures fed high-CT substrate; high-CT cultures treated with penicillin + streptomycin were of intermediate BCVFA concentration (0.8, 0.6, and 0.7 mM, respectively) and not different from either antimicrobial-free or cycloheximide-treated cultures fed high-CT substrate. Compared with tannin-free substrate, BCVFA concentration was 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 may have partially compensated for that by bacteria when bacterial activities were suppressed. Hassanat and Benchaar (2012) reported that BCVFA concentrations were not affected by the presence of condensed tannins at doses comparable to those used in our

study; however, BCVFA concentrations increased, relative to tannin-free cultures at CT doses lesser than those used in our study (i.e., 20 and 50 g CT / kg substrate).

Condensed tannins had general suppressive effects on 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. Prior dietary exposure of ruminal-fluid donors to CT mitigated the negative effects of dietary CT tannins to some extent. In the presence of CT, total VFA and propionate concentrations were greater in cultures with exposed-animal inoculum than in cultures with non-exposed animal inoculum. Cultures that contained penicillin + streptomycin had lesser total gas production, total VFA, acetate, propionate, butyrate, valerate, and branched-chain VFA concentrations than antimicrobial-free cultures or cultures spiked with cycloheximide. Under the conditions of our study, bacterial suppression markedly decreased total and individual VFA concentrations, whereas fungal suppression produced results similar to non-suppressed cultures. We concluded that activities of ruminal fungi, under the conditions of our study, contributed little to major fermentation parameters during a 48-h *in vitro* incubation compared to those of bacteria.

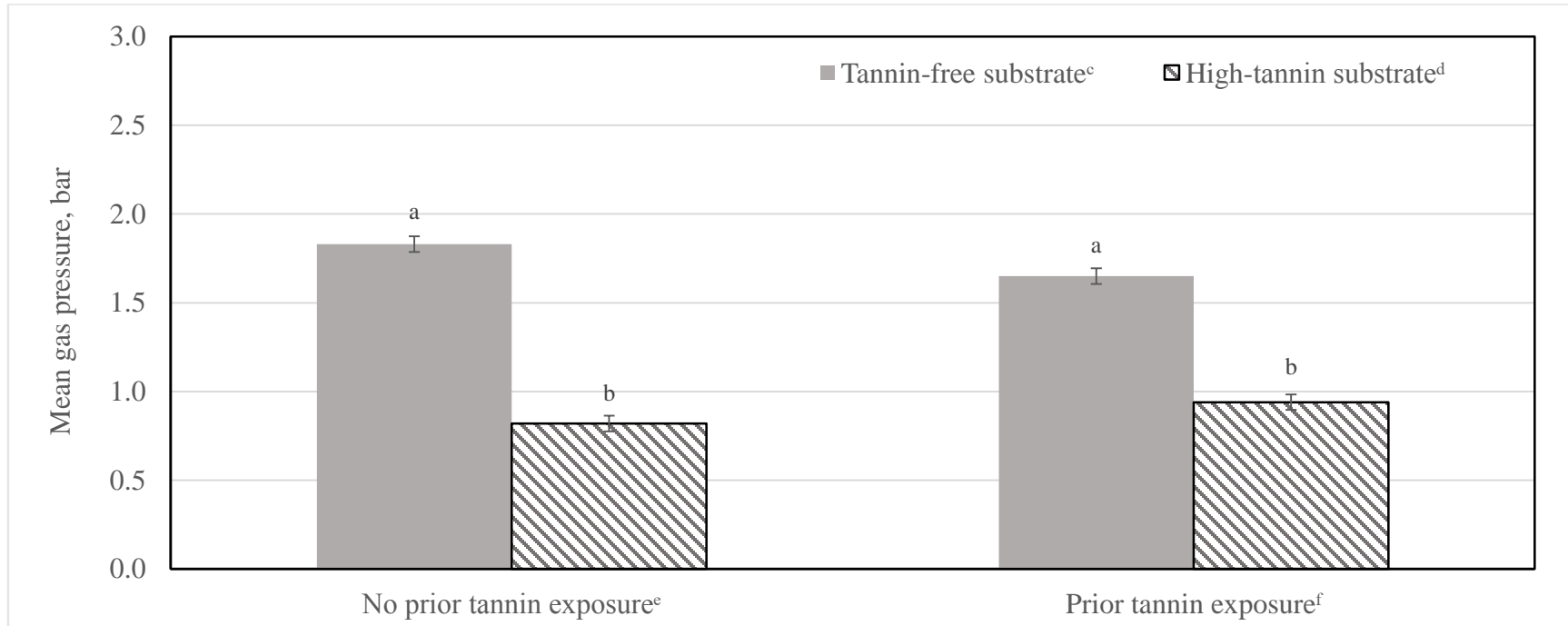
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Figure 2.1. Effects of culture substrate and prior dietary tannin exposure on gas pressure following a 48-h *in vitro* incubation.



^{a, b} Means with unlike superscripts differ ($P < 0.001$; F -test protected LSD = 0.21).

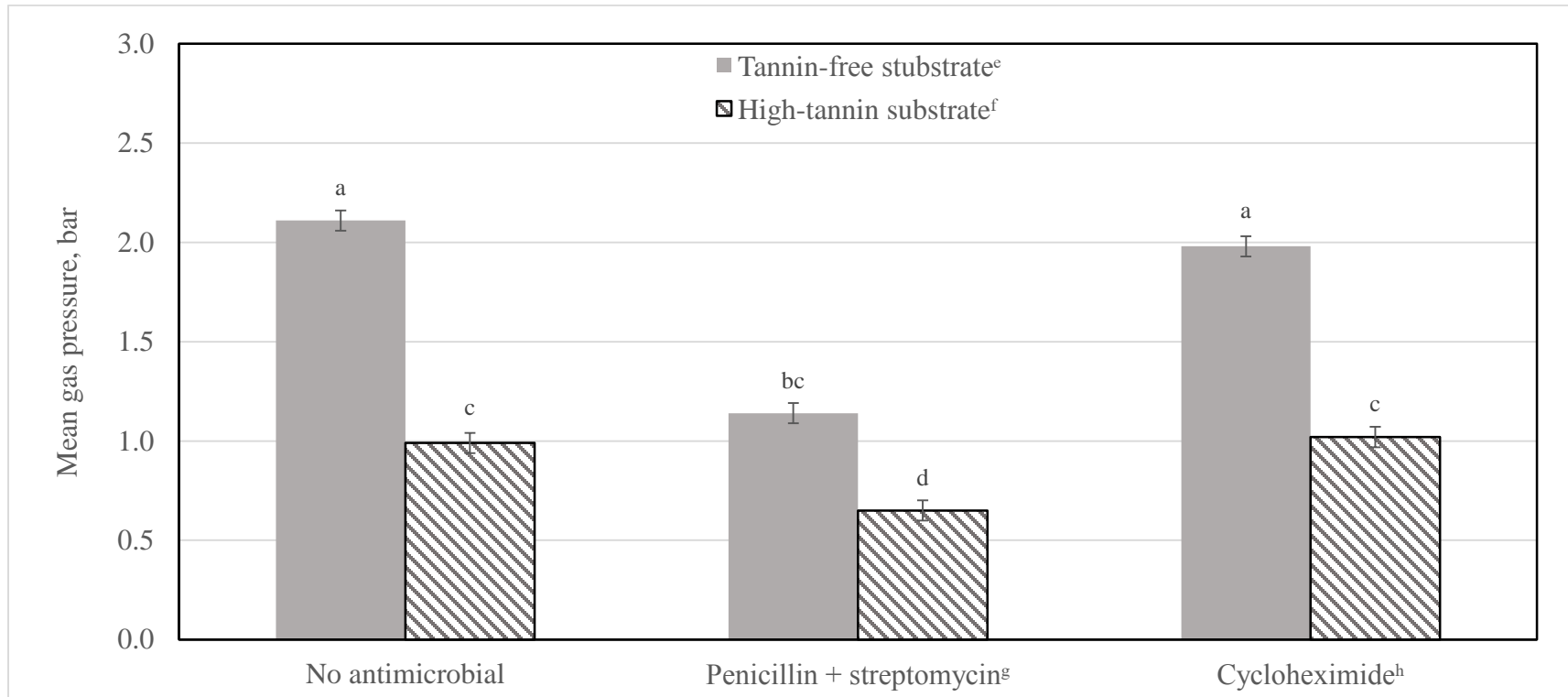
^c Culture substrate consisted of ground smooth bromegrass hay only.

^d Culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^e Cultures contained ruminal fluid collected from animals fed a tannin-free diet.

^f Cultures contained ruminal fluid collected from animals fed a high-tannin diet.

Figure 2.2. Effects of culture substrate and antimicrobial on gas pressure following a 48-h *in vitro* incubation.



^{a, b, c, d} Means with unlike superscripts differ ($P < 0.001$; F -test protected LSD = 0.24).

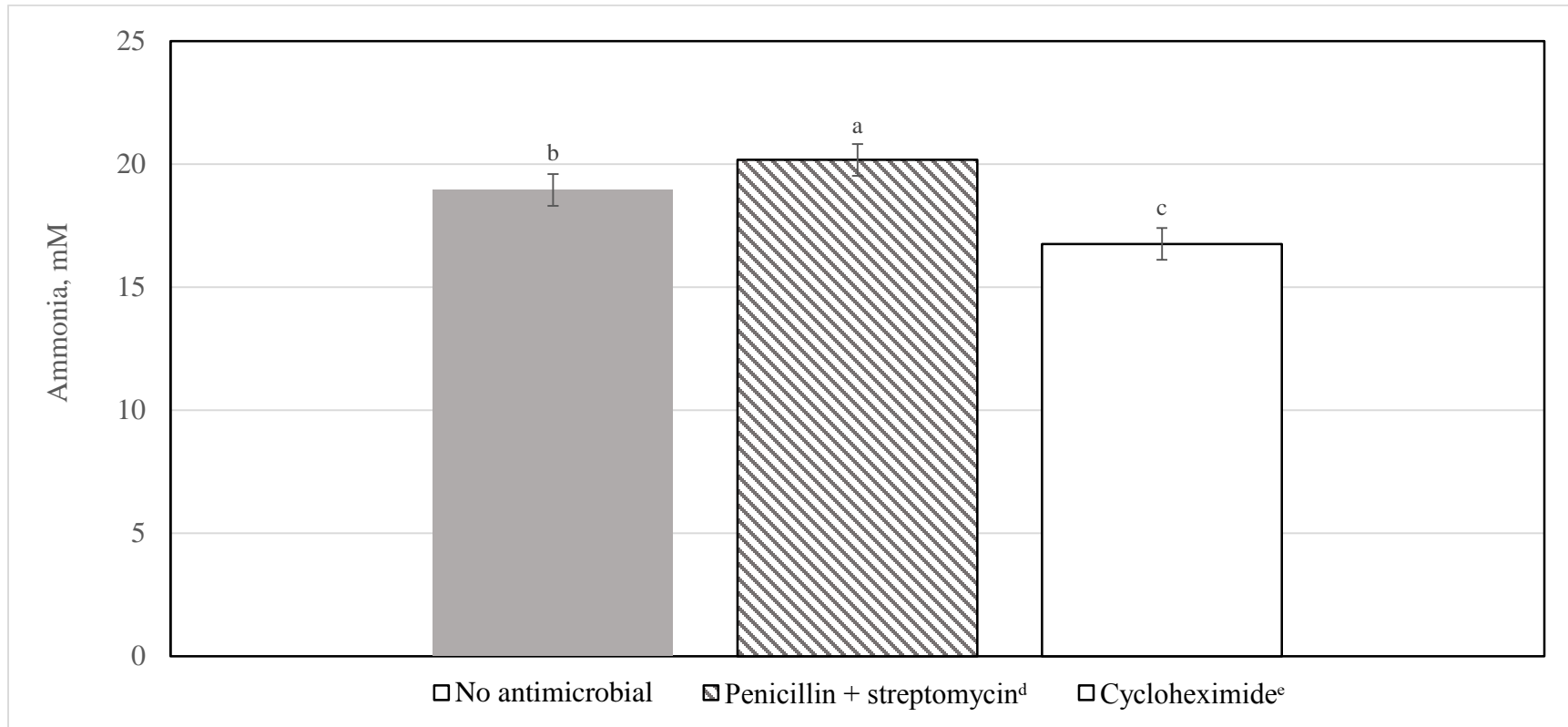
^e Culture substrate consisted of ground smooth brome grass hay only.

^f Culture substrate consisted of 89.8% ground smooth brome grass hay and 10.2% condensed tannins of plant origin.

^g Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^h Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Figure 2.3. Main effect of antimicrobial on ammonia concentration following a 48-h *in vitro* incubation.

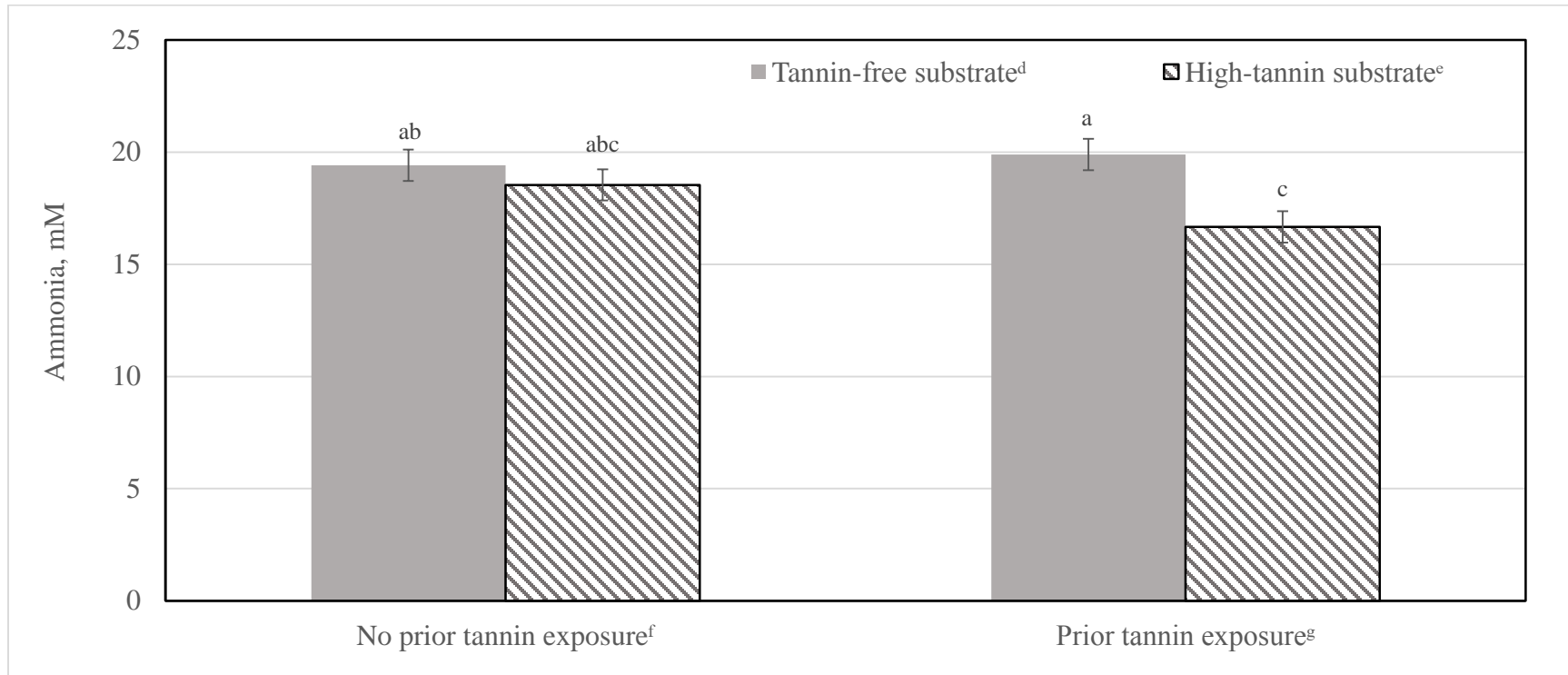


^{a, b, c} Means with unlike superscripts differ ($P < 0.001$; F -test protected LSD = 1.19).

^d Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^e Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Figure 2.4. Effects of culture substrate and prior dietary tannin exposure on ammonia concentration following a 48-h *in vitro* incubation.



^{a, b, c} Means with unlike superscripts differ ($P < 0.001$; F -test protected LSD = 2.05).

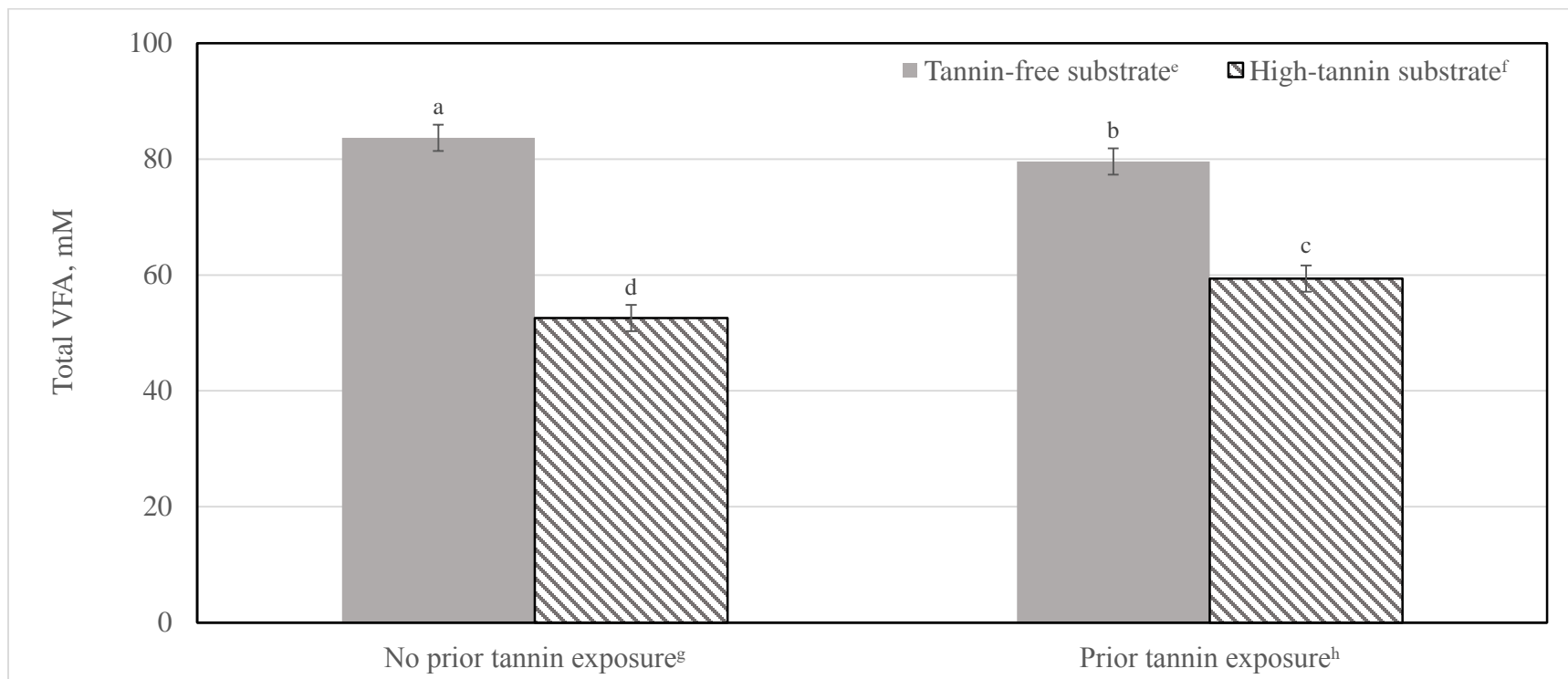
^d Culture substrate consisted of ground smooth brome grass hay only.

^e Culture substrate consisted of 89.8% ground smooth brome grass hay and 10.2% condensed tannins of plant origin.

^f Cultures contained ruminal fluid collected from animals fed a tannin-free diet.

^g Cultures contained ruminal fluid collected from animals fed a high-tannin diet.

Figure 2.5. Effects of culture substrate and prior dietary tannin exposure on total VFA concentration following a 48-h *in vitro* incubation.



^{a, b, c, d} Means with unlike superscripts differ ($P < 0.001$; F -test protected LSD = 3.43).

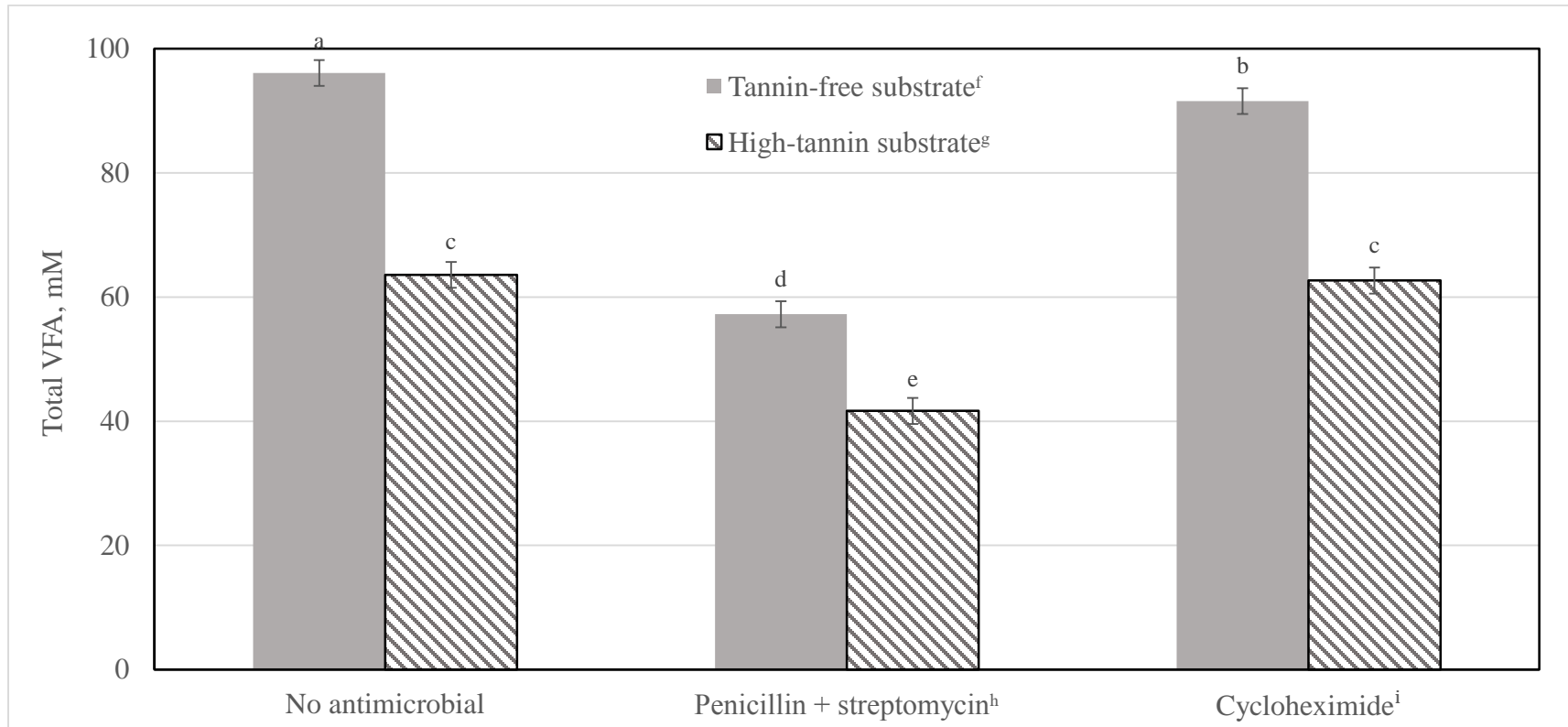
^e Culture substrate consisted of ground smooth bromegrass hay only.

^f Culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^g Cultures contained ruminal fluid collected from animals fed a tannin-free diet.

^h Cultures contained ruminal fluid collected from animals fed a high-tannin diet.

Figure 2.6. Effects of culture substrate and antimicrobial on total VFA concentration following a 48-h *in vitro* incubation.



^{a, b, c, d, e} Means with unlike superscripts differ ($P < 0.001$; F -test protected LSD = 3.43).

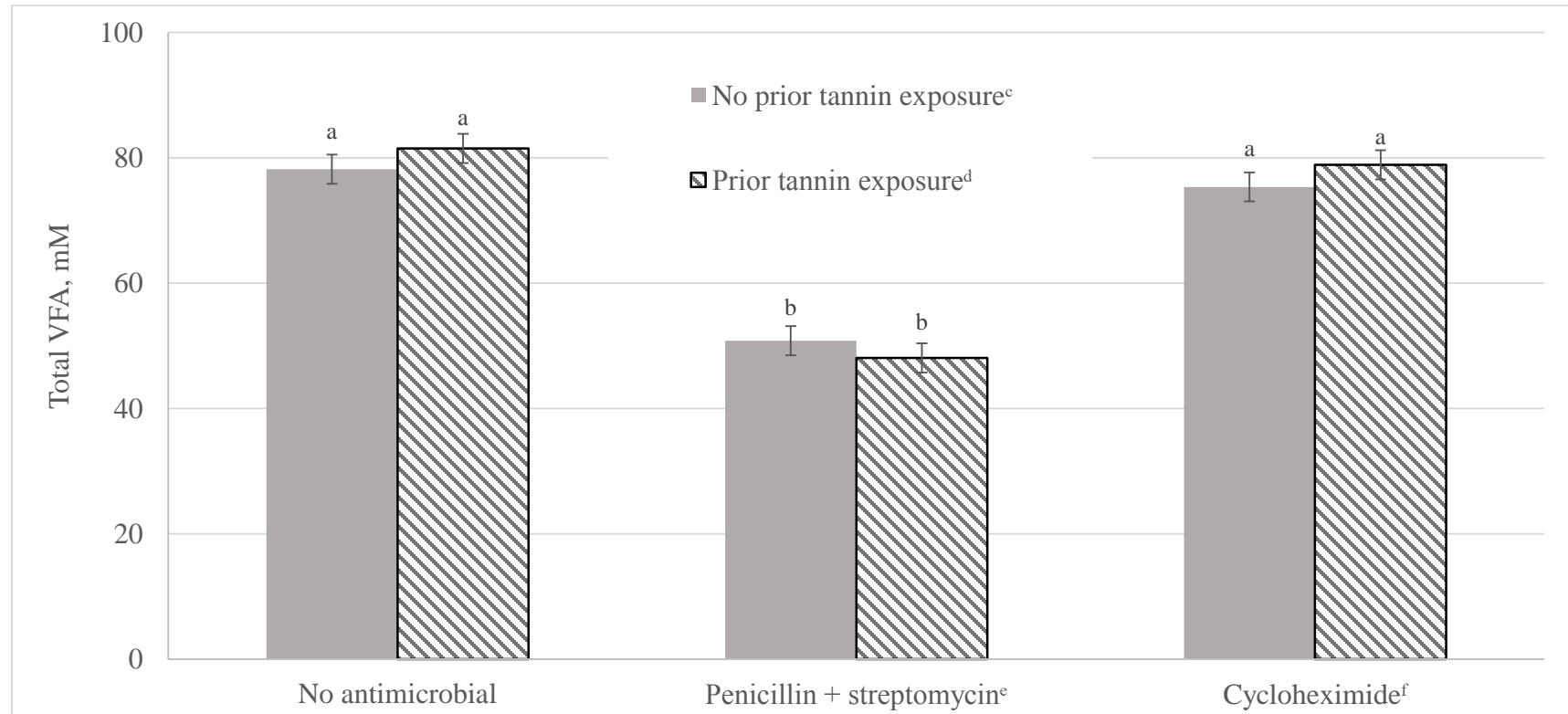
^f Culture substrate consisted of ground smooth bromegrass hay only.

^g Culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^h Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

ⁱ Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Figure 2.7. Effects of antimicrobial and prior dietary tannin exposure on total VFA concentration following a 48-h *in vitro* incubation.



^{a, b} Means with unlike superscripts differ ($P = 0.001$; F -test protected LSD = 8.51).

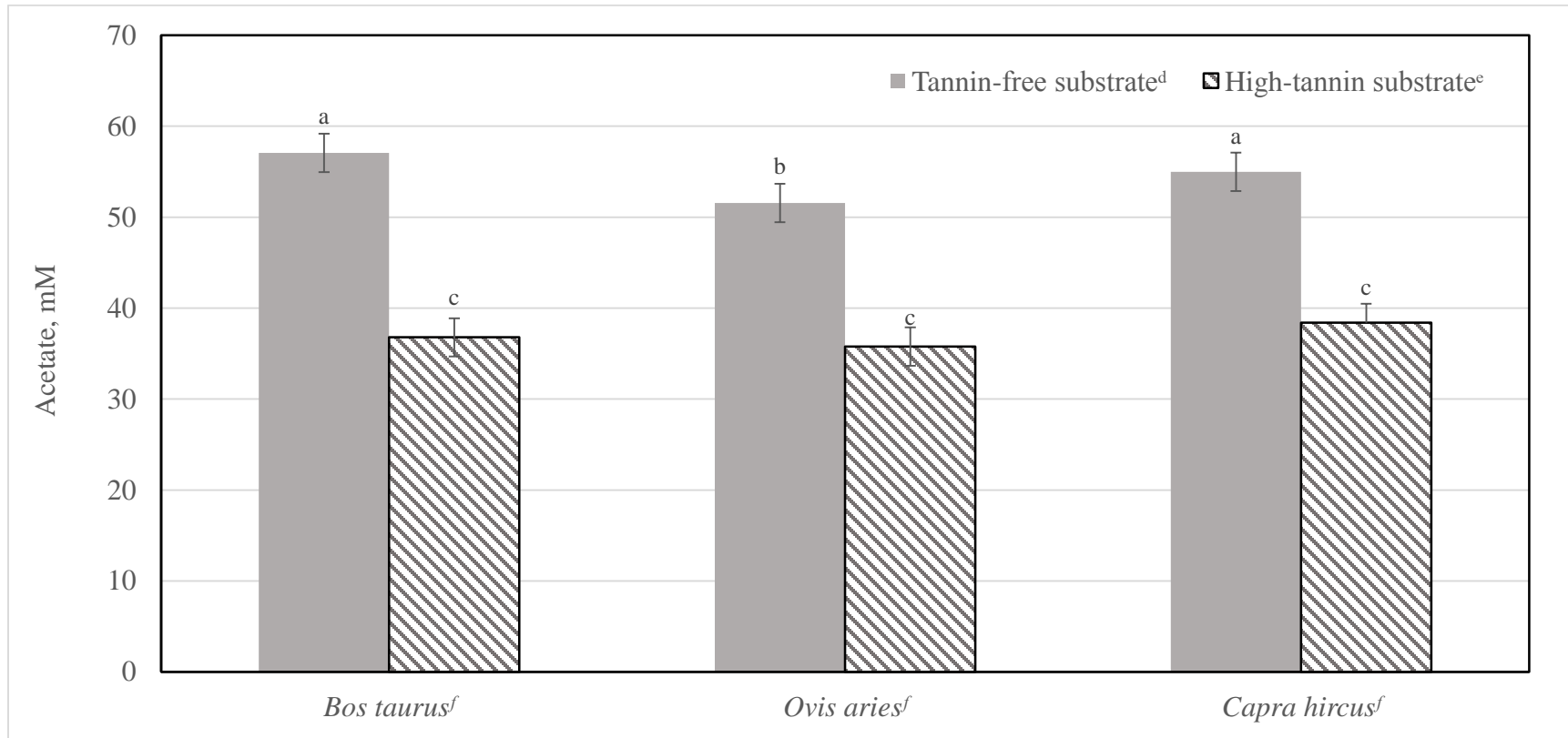
^c Culture substrate consisted of ground smooth bromegrass hay only.

^d Culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^e Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^f Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Figure 2.8. Effects of donor-species inoculum and culture substrate on acetate concentration following a 48-h *in vitro* incubation.



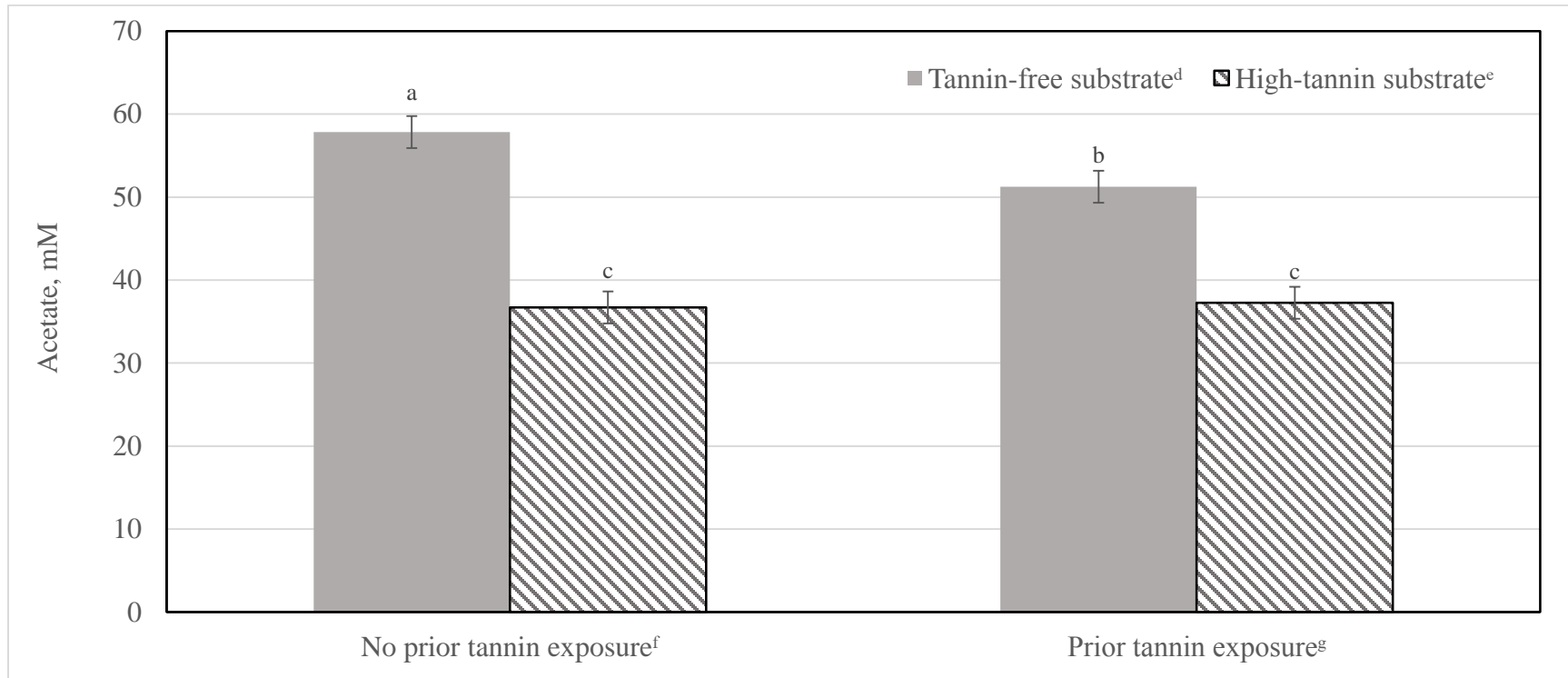
^{a, b, c} Means with unlike superscripts differ ($P < 0.001$; F -test protected LSD = 2.96).

^d Culture substrate consisted of ground smooth bromegrass hay only.

^e Culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^f $n = 3$ /species.

Figure 2.9. Effects of culture substrate and prior dietary tannin exposure on acetate concentration following a 48-h *in vitro* incubation.



^{a, b, c} Means with unlike superscripts differ ($P < 0.001$; F -test protected LSD = 5.89).

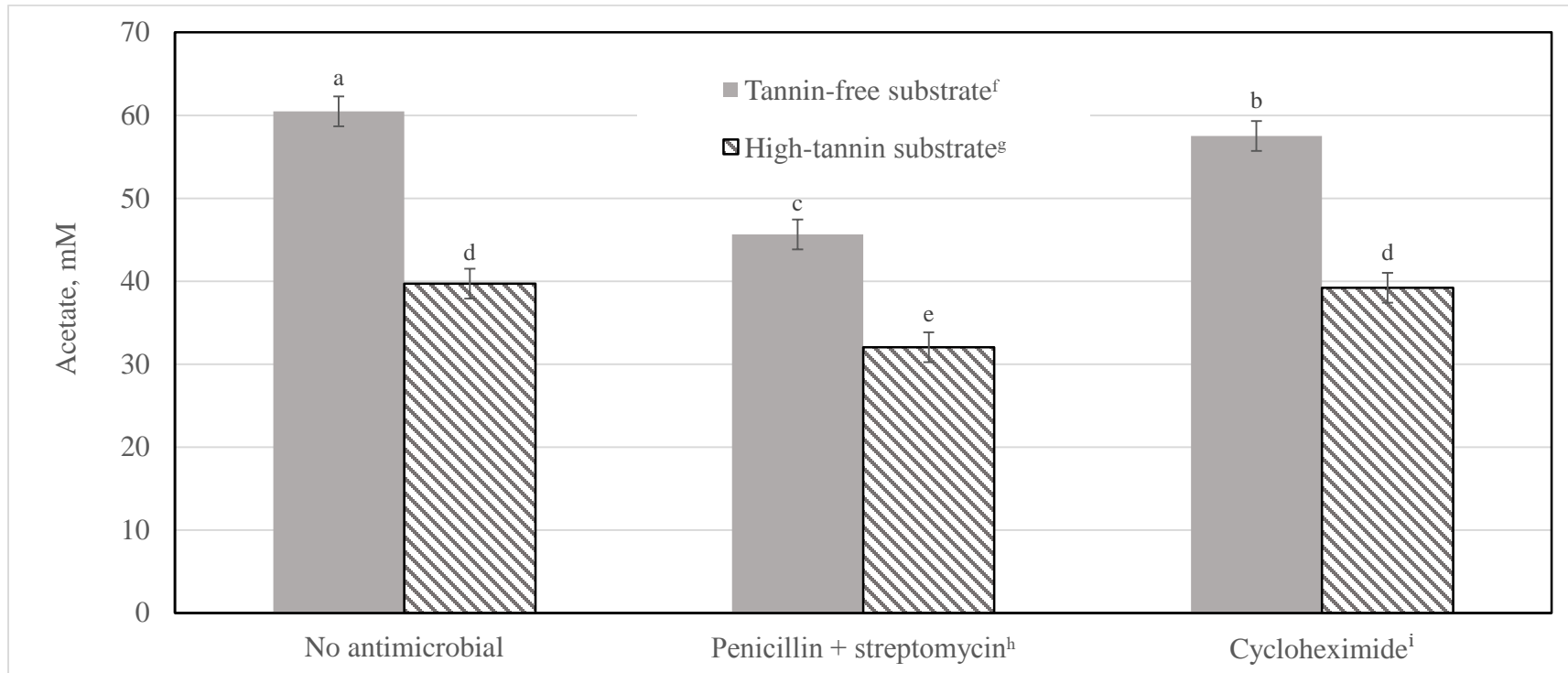
^d Culture substrate consisted of ground smooth bromegrass hay only.

^e Culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^f Cultures contained ruminal fluid collected from animals fed a tannin-free diet.

^g Cultures contained ruminal fluid collected from animals fed a high-tannin diet.

Figure 2.10. Effects of culture substrate and antimicrobial on acetate concentration following a 48-h *in vitro* incubation.



^{a, b, c, d, e} Means with unlike superscripts differ ($P < 0.001$; F -test protected LSD = 2.96).

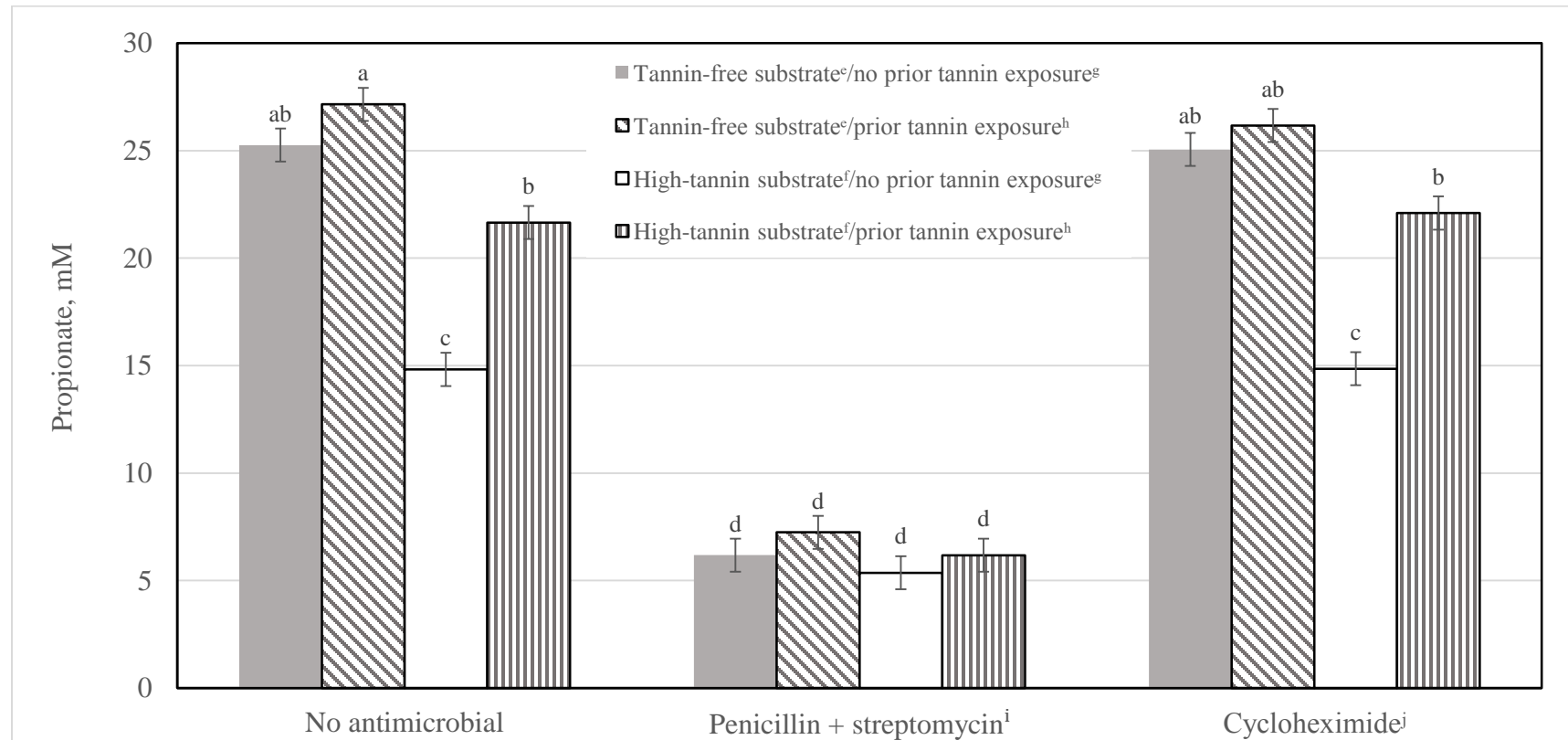
^f Culture substrate consisted of ground smooth bromegrass hay only.

^g Culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^h Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

ⁱ Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Figure 2.11. Effects of culture substrate, antimicrobial, and prior dietary tannin exposure on propionate concentration following a 48-h *in vitro* incubation.



^{a, b, c, d} Means with unlike superscripts differ ($P < 0.001$; F -test protected LSD = 3.77).

^e Culture substrate consisted of ground smooth bromegrass hay only.

^f Culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

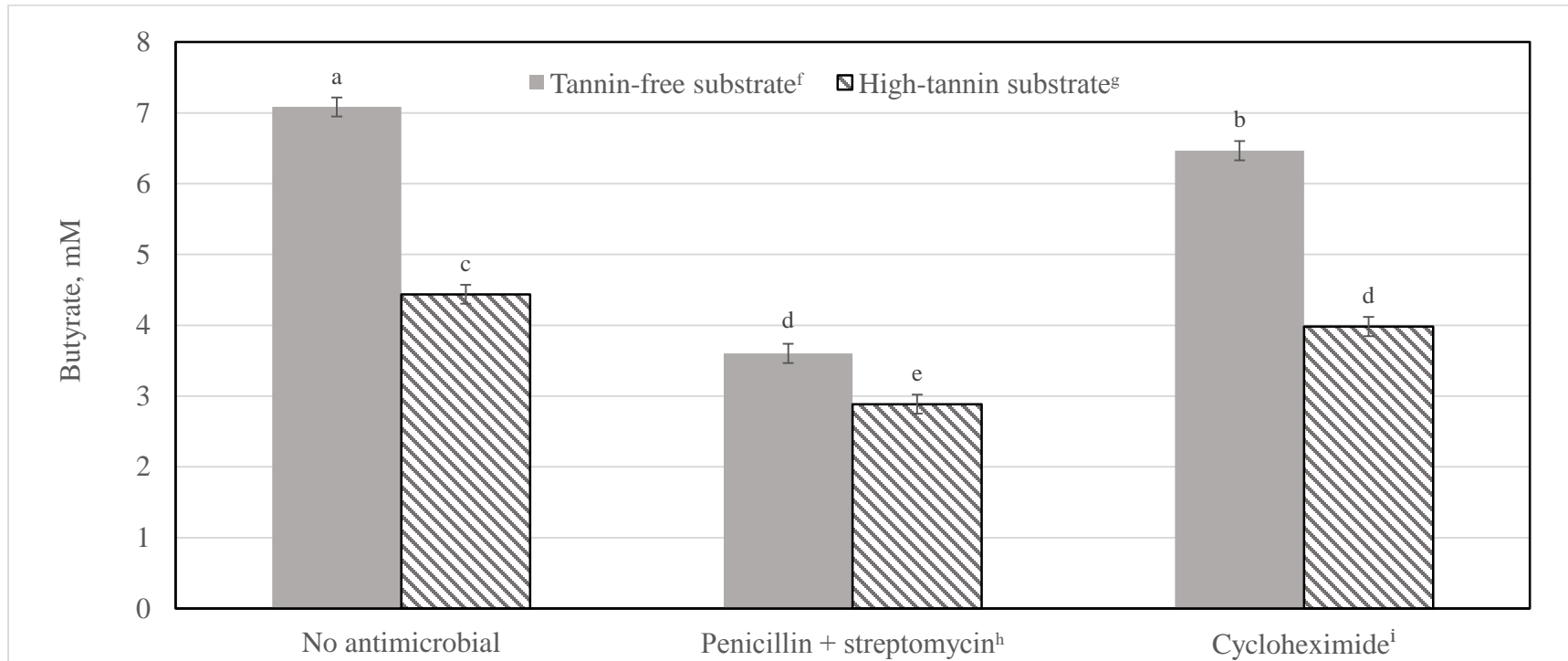
^g Cultures contained ruminal fluid collected from animals fed a tannin-free diet.

^h Cultures contained ruminal fluid collected from animals fed a high-tannin diet.

ⁱ Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^j Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Figure 2.12. Effects of culture substrate and antimicrobial on butyrate concentration following a 48-h *in vitro* incubation.



^{a, b, c, d, e} Means with unlike superscripts differ ($P < 0.001$; F -test protected LSD = 0.389).

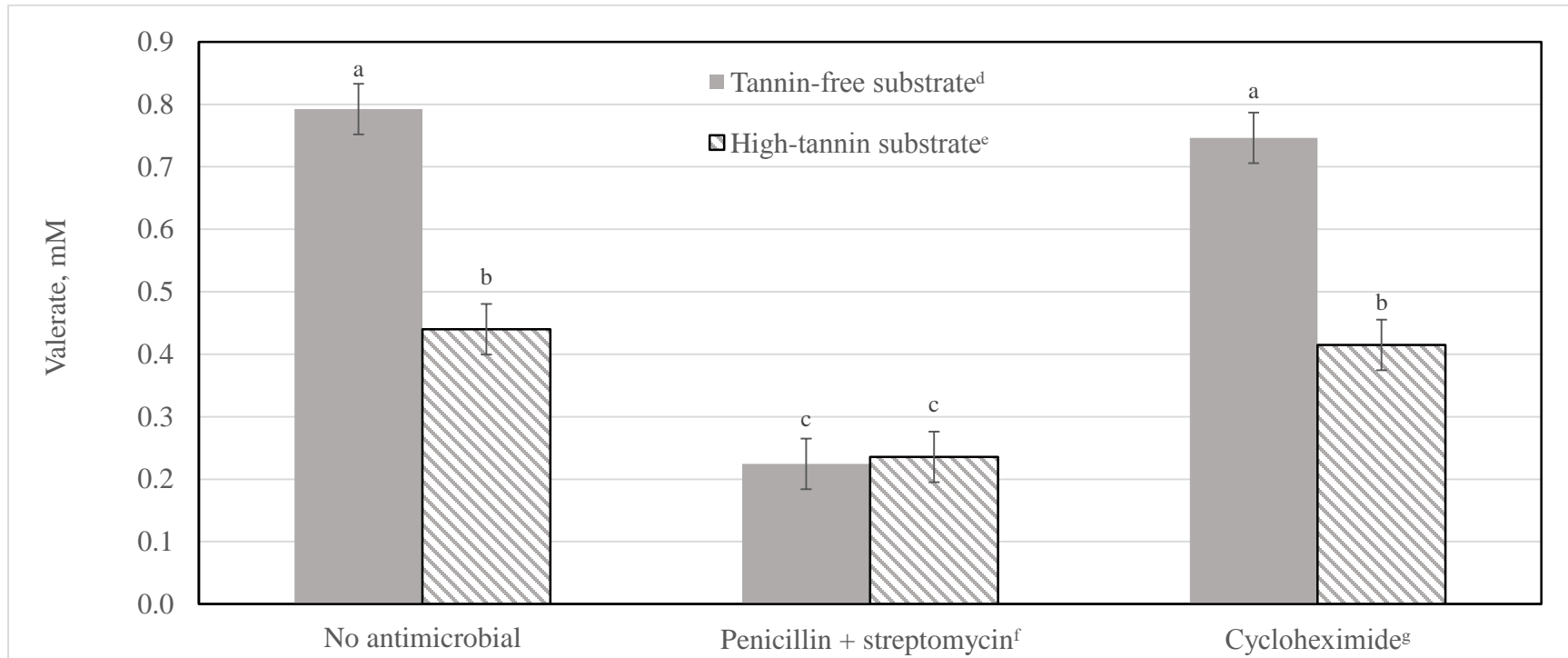
^f Culture substrate consisted of ground smooth bromegrass hay only.

^g Culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^h Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

ⁱ Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Figure 2.13. Effects of culture substrate and antimicrobial on valerate concentration following a 48-h *in vitro* incubation.



^{a, b, c} Means with unlike superscripts differ ($P < 0.001$; F -test protected LSD = 0.160).

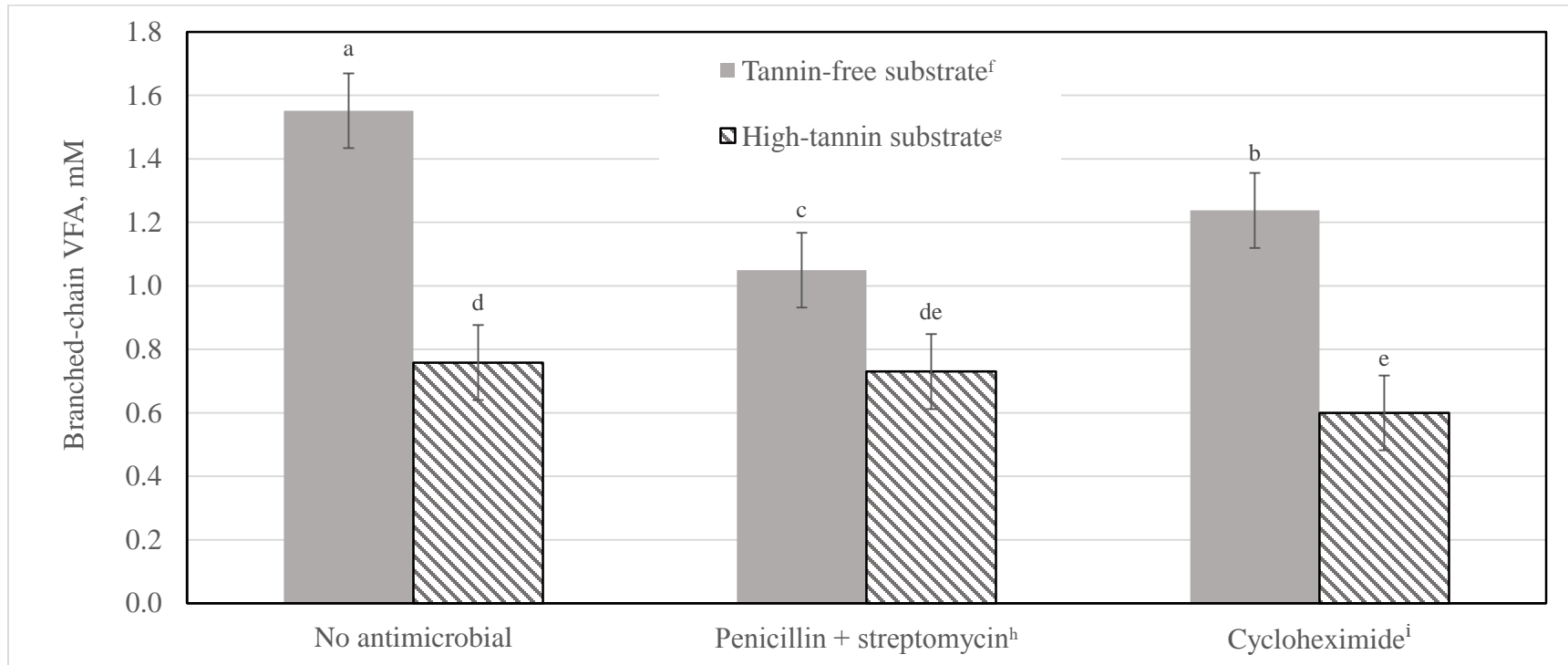
^d Culture substrate consisted of ground smooth bromegrass hay only.

^e Culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^f Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^g Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Figure 2.14. Effects of culture substrate and antimicrobial on branched-chain VFA (isobutyrate + isovalerate) concentration following a 48-h *in vitro* incubation.



^{a, b, c, d, e} Means with unlike superscripts differ ($P < 0.001$; F -test protected LSD = 0.148).

^f Culture substrate consisted of ground smooth bromegrass hay only.

^g Culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^h Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

ⁱ Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Chapter 3 – Effects of High-Tannin Substrate, Prior Dietary Tannin Exposure, Antimicrobial Inclusion, and Animal Species on IVDMD, Ammonia Concentration, Total VFA Concentration, and Individual VFA Concentrations During a 48-h *In Vitro* Incubation

Abstract

Effects of prior dietary condensed-tannin (CT) exposure, dietary CT, animal species, and antimicrobial additive on rate of *in vitro* fermentation were measured at 4, 8, 12, 24, 36, and 48 h of incubation. Cows, sheep, and goats ($n = 3$ / species) were used in a 2-period, randomized complete-block experiment with a $2 \times 3 \times 2 \times 3$ factorial arrangement of treatments. Factor 1 was culture substrate: high-CT or tannin-free. Factor 2 was inoculum-donor species: *Bos taurus*, *Ovis aries*, or *Capra hircus*. Factor 3 was donor species exposure to a high-CT diet: non-exposed or exposed. Factor 4 was antimicrobial additive: none, bacterial suppression (penicillin + streptomycin), or fungal suppression (cycloheximide). Tannin-free or high-CT substrates were incubated *in vitro* using ruminal fluid from animals either not exposed to dietary CT (period 1) or exposed to dietary CT (period 2). Periods consisted of an adaptation to tannin-free (10 d) or high-CT diets (21 d) and a 15-d period of ruminal-fluid collection via stomach tube. The extent of IVDMD after 48 h of incubation was greater ($P < 0.001$; LSD = 3.69) for cultures with tannin-free substrate than for cultures with high-CT substrate (25.1 and 16.5%, respectively). Ammonia concentration was influenced ($P < 0.00$; LSD = 4.01) by donor-animal species, culture substrate, antimicrobial, and donor-animal tannin-exposure status. Suppression of the fermentative activities of bacteria in the presence of dietary CT resulted in diminished ammonia accumulation without prior CT exposure, but not when donor inoculum was CT-exposed. Effects of substrate

type on total VFA and individual VFA concentrations were influenced ($P < 0.001$) by addition of antimicrobials and by time. Tannin-free cultures without antimicrobial and tannin-free cultures spiked with cycloheximide had greater total VFA and individual VFA concentrations than other treatments at 36 and 48 h of incubation. Cultures with high-CT substrate or cultures in which bacterial fermentative activities were suppressed with penicillin + streptomycin produced similar degrees of depression in total VFA and individual VFA concentrations. We interpreted these data to indicate that: 1) dietary CT had strong negative effects on VFA concentrations and 2) bacterial fermentative activities were of greater importance to VFA concentrations than fungal fermentative activities, in the presence or absence of dietary CT.

Key words: condensed tannins, dietary tannin adaptation, *in vitro* fermentation

Introduction

Condensed tannins (CT) are phenolic compounds found in a wide variety of plants and are a functional defense mechanism against stress, disease, and herbivory (Min et al., 2003). Condensed tannins limit DM intake and ruminal protein and DM digestibility by ruminants *in vivo* (Makkar, 2003; Waghorn, 2008; Eckerle, 2011); CT complex with proteins and structural carbohydrates via hydrogen and hydrophobic bonding and render them unavailable to host-animal digestive processes (Wroblewski et al., 2001). Most CT-protein complexes form between pH 3.5 to 7 (i.e., normal ruminal pH) and dissociate at a pH less than 3.5 (i.e., normal abomasal pH; Min et al., 2003) allowing post-ruminal protein digestion by the host animal; however, a sudden dearth in ruminally-available N causes a general malaise in grazing herbivores that can rapidly decrease consumption of high-CT forages (Eckerle et al., 2011).

Decreased ammonia, total VFA, butyrate, valerate, and branched-chain VFA concentrations have been reported in high-CT *in vitro* cultures (Hassanat and Benchaar, 2012).

Diets containing CT decreased ruminal protein degradation *in vitro* (Hassanat and Benchaar, 2012) and *in vivo* (Al-Dobaib, 2009). Additionally, CT suppressed growth rates of several vital ruminal-bacterial populations (Min et al., 2005). Generally, beef cattle have a lower tolerance for high-CT diets than small ruminants. Frutos et al. (2004) reported that *in vitro* DM disappearance was less for cattle and sheep than for goats and deer when CT were included in culture media. Insufficient research has focused on adaptation of various ruminant species to dietary CT. In addition, the specific effects of dietary CT on ruminal fungal and ruminal bacterial populations are unknown. Therefore, our objective was to evaluate the influences of prior exposure to dietary CT in *Bos taurus*, *Ovis aries*, and *Capra hircus*, with and without selective suppression of either ruminal bacteria or ruminal fungi, on *in vitro* DM disappearance and concentrations of ammonia, total VFA, and individual VFA during a time-series 48-h *in vitro* incubation.

Materials and Methods

Study Preparation

The Kansas State University Institutional Animal Care and Use Committee reviewed and approved all animal-handling and animal-care practices used in this research (protocol no. 3423). Three beef cattle (551 ± 30 kg BW), 3 sheep (68 ± 3 kg BW), and 3 goats (49 ± 4 kg BW) were used in this experiment. Sheep and goats were housed together in a 10 x 10 m pen and cattle were housed in an adjacent 100 x 100 m pen. Smooth brome grass hay (*Bromus inermis*; 87.9% DM, 9.1% CP, 76.2% NDF, and 47.0% ADF) was offered to all animals daily in round-bale feeders (diameter = 2.5 m) in amounts calculated to allow *ad libitum* intake ($\approx 4.0\%$ BW/d). One animal from each species was assigned randomly to 1 of 3 cohorts; cohorts were assigned randomly to 1 of 3 sampling times during each of 2 experimental periods. Animals were fed a single tannin-free diet during period 1 and a single tannin-containing diet during period 2.

Tannin-free and tannin-containing substrates were subject to *in vitro* fermentation using ruminal inoculum harvested from *Bos taurus*, *Ovis aries*, and *Capra hircus* that were either not exposed to dietary CT (period 1) or exposed to dietary CT (period 2).

Adaptation to Tannin-Free Diets

The timeline of our experiment was expressed relative to the first day of animal adaptation (d 1) to treatment diets. All animal cohorts were fed tannin-free smooth bromegrass hay *ad libitum* for 10 d to begin period 1. Ruminal fluid was collected via stomach tube from animals from d 19 to 25 for use in *in vitro* cultures. Ruminal fluid was collected from cohort 1 on d 19, from cohort 2 on d 22, and from cohort 3 on d 25. During the period of ruminal fluid collection, animals continued to be fed for *ad libitum* intake of smooth bromegrass hay, as during the adaptation phase of the experiment. All animals had unrestricted access to fresh water, a salt block (98.0% NaCl; Compass Minerals, Chicago, IL) and a mineral block (95.5% NaCl, 3500 ppm Zn, 2000 ppm Fe, 1800 ppm Mn, 280 ppm Cu, 100 ppm I, and 60 ppm Co; Compass Mineral, Chicago, IL) during period 1. The tannin-free diet was assumed to allow no exposure to CT.

Adaptation to High-Tannin Diets

Animal subjects were adapted to high-CT intake conditions by providing them with grain-byproduct supplements that were spiked with quebracho tannins during period 2. Hassanat and Benchaar (2012) reported that CT extracted from certain trees in the *Anacardiaceae* and *Apocynaceae* families, known commonly as quebracho trees, had elevated concentrations of CT which produced dose-dependent effects on ruminal digestion parameters *in vitro*. Purified, feed-grade quebracho-tannin extract (QT) was procured from Wintersun Chemical (Ontario, CA) for use in CT-containing supplements and CT-containing culture substrates.

To begin period 2, animal subjects were fed smooth brome grass hay *ad libitum* for 21 d. Each animal was also individually fed a supplement which contained QT at 0.1% of BW/d, soybean hulls at 0.2% of BW/d, and dried molasses at 0.05% of BW/d. Soybean hulls and molasses were fed to encourage complete consumption of the prescribed dose of QT. The animals were penned individually each morning (0730) and each evening (2000) 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 DMI. Supplement consumption by *Ovis aries* and *Capra hircus* was complete each day. *Bos taurus* left an average of 183 ± 10 g QT unconsumed daily, a consumption level equivalent to only 67% of the targeted dose. This agreed with Eckerle et al. (2011) who reported a strong aversion among beef cows to dietary CT. All animal subjects had unrestricted access to fresh water, a salt block (98.0% NaCl; Compass Minerals, Chicago, IL), and a mineral block (95.5% NaCl, 3500 ppm Zn, 2000 ppm Fe, 1800 ppm Mn, 280 ppm Cu, 100 ppm I, 60 ppm Co; Compass Minerals, Chicago, IL) during period 2.

Ruminal fluid was collected via stomach tube from d 31 to 37 from animals for *in vitro* cultures during period 2. Ruminal fluid was collected from cohort 1 on d 31, from cohort 2 on d 34, and from cohort 3 on d 37. During the period of ruminal fluid collection, animals continued to be fed for *ad libitum* intake of smooth brome grass hay, as during the adaptation phase of the experiment; moreover, they continued to be fed CT-containing supplements daily.

Adaptation to the high-CT diet was considered to provide prior exposure to CT. Although our measurements of the effects of tannin-free and high-CT diets were separated in time, we assumed that the time difference did not confound our comparisons. Animal subjects, facilities,

and the conditions under which *in vitro* techniques were used during periods 1 and 2 were held constant.

Ruminal Fluid Collection

Ruminal fluid was collected orally at 0730 on the schedule designated for each animal cohort. A simple, hand-made vacuum strainer was used for this purpose (see chapter 2 of this document for a full description of the device). Ruminal fluid was collected from each animal in a cohort in the following order: *Bos taurus*, *Ovis aries*, and then *Capra hircus*. *Bos taurus* were restrained using a chute with a locking head gate. *Ovis aries* and *Capra hircus* were restrained manually. An oral speculum was inserted into the mouth (15 cm for *Bos taurus* and 8 cm for *Ovis aries* and *Capra hircus*) to prevent animals from biting the collection tube, thereby halting fluid flow or damaging the tube. To prevent excessive salivary contamination, the first 200 mL of ruminal fluid collected from each animal was discarded. Approximately 700 mL of fluid was harvested from each animal at each collection; collection time was approximately 7 min / animal. Harvested ruminal fluid from each animal was transferred immediately to a single 3-L, pre-warmed, insulated bottle for transport to laboratory facilities. Bottles were warmed by filling them with 39°C deionized H₂O approximately 1 h before ruminal-fluid collection began. The water was discarded immediately prior to transfer of the ruminal fluid. Upon arrival at laboratory facilities, harvested ruminal fluid was strained through 8 layers of cheesecloth and poured into a separatory flask; it was allowed to separate for 30 min. The heaviest fraction, containing protozoa and other waste, was discarded (ANKOM, 2009).

McDougall's artificial saliva was prepared 24 h before each ruminal-fluid collection and stored in an incubator at 39°C. One liter of McDougall's artificial saliva included the following: 9.80 g NaHCO₃, 3.71 g Na₂HPO₄, 0.57 g KCl, 0.47 g NaCl, 0.12 g MgSO₄•7H₂O, 0.80 g

NH₂CONH₂, and 0.04 g CaCl₂. Four L of McDougall's artificial saliva were prepared for each ruminal-fluid collection. All of the ingredients, with the exception of CaCl₂, were added to 1-L volumetric flasks and the flasks half-filled with deionized H₂O. Ingredients were dissolved via agitation and the flasks were then filled to the volume line.

Immediately before each ruminal-fluid collection, the CaCl₂ was added to the McDougall's artificial saliva and dissolved. The flasks were then placed in an incubator at 39°C. Nitrogen gas was concurrently bubbled through the McDougall's artificial saliva and the pH adjusted to 6.8 to 7.0, using aqueous phosphoric acid (85% H₃PO₄). Ruminal fluid and McDougall's artificial saliva were included in the cultures at a 1:2 ratio (McDougall, 1948).

Culture Substrate Preparation

During periods 1 and 2, the tannin-free substrate added to ruminal inoculum of *Bos taurus*, *Ovis aries*, and *Capra hircus* was the same as the smooth bromegrass hay with which animals were fed (87.9% DM; 9.1% CP, 76.2% NDF, and 47.0% ADF). Hay was dried at 55°C for 48 h and ground (#4 Wiley Mill, Thomas Scientific, Swedesboro, NJ, USA) to pass a 1-mm screen before it was added to the culturing devices. The tannin-contaminated substrate added to ruminal inoculum of *Bos taurus*, *Ovis aries*, and *Capra hircus* during periods 1 and 2 was a composite of the smooth bromegrass hay used as the tannin-free substrate and CT in the form of QT. Hay and QT were blended to achieve a substrate CT concentration of 10.2% (wt/wt; Eckerle et al., 2010). 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, 0.5% NDF, and 2.7% ADF.

In order to determine the relative importance of bacterial and fungal fermentative activities *in vitro*, tannin-free and tannin-contaminated substrates were subject to *in vitro*

fermentation with 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., Milwaukee, WI). A bacterial-suppression solution was prepared, containing 12.5 mg of penicillin G and 2 mg of streptomycin sulfate per mL of deionized water. One mL of that solution was added to *in vitro* cultures per 10 mL of ruminal fluid. Fungal suppression was accomplished by preparing a solution of 5 mg of cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) per mL of deionized water. One mL of fungal-suppression solution was added to *in vitro* cultures per 10 mL of ruminal fluid. Unsuppressed (i.e., no added antimicrobial) *in vitro* cultures were examined concurrently for reference.

In Vitro Rate of Digestion

In vitro rate of digestibility, VFA concentrations, and NH₃ concentrations were measured using a 48-h time-series, *in vitro* 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 the dry tube weight. Ammonia concentrations, VFA concentrations, and IVDMD were measured after incubation periods of 4, 8, 12, 24, 36, and 48 h.

Each treatment was represented by 6 tubes (1 / time point); moreover, 18 blank tubes per animal species (i.e., containing ruminal inoculum but no substrate; 3 / time point, 1 for each antimicrobial treatment) were incubated during each *in vitro* trial. Twenty-four h before ruminal fluid collection, tannin-free substrate (0.5 g) was added to each tube, excepting those designated

as blanks. Quebracho tannin (0.0794 g) was also added to each tube designated for high-CT substrate at that time.

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 N₂ 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 endpoint, one tube from each treatment × species combination (n = 6) and 3 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 × g for 15 min and then placed back on ice. Ammonia and VFA samples were collected by transferring 1 mL of fluid from each tube into 2 separate 2-mL conical vials, into which 0.25 mL of aqueous metaphosphoric acid (25%, w/v) had been added to suspend microbial activity. An additional 4 mL of fluid from each tube was transferred to a separate 5-mL tube with 1 mL of aqueous metaphosphoric acid (25%, w/v). Fluid samples were frozen pending subsequent analysis. The tubes, with residual substrate and fluid, were placed in an oven at 105°C for 48 h; tubes with dry samples were placed in a desiccator to cool and then weighed to determine IVDMD.

Analytical Procedures

In vitro DM disappearance (IVDMD) was determined using procedures described by Smith et al. (2013). IVDMD was calculated as follows:

$$\frac{\text{initial DM sample wt} - [(\text{final DM sample wt} - \text{empty tube dry wt}) - (\text{average blank dry wt} - \text{empty tube dry wt})]}{\text{initial DM sample wt}}$$

Before VFA analysis, samples were thawed for 30 min, agitated for 10 s with a vortex mixer, and centrifuged at $16,000 \times g$ for 10 min (Eppendorf model 5415C; Brinkmann Instruments, Inc., Westbury, NY). The supernatant from each sample was transferred to a 2-mL gas-chromatography vial. Sample concentrations of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid were measured via gas chromatography. A 1.8 m x 6 mm, 4-mm i.d. glass column packed with GP 10% SP-1200 / 1% H₃PO₄ (Supelco #1-1965; Sigma-Aldrich) was used for analyses. Nitrogen was the carrier gas (flow rate = 80 mL/min); detection was by flame ionization (compressed air flow = 200 mL/min, H₂ flow = 20 mL/min, combined flow = 200 mL/min). The injection and detector were set at 250°C and the column temperature was maintained between 120 and 140°C.

Ammonia samples were thawed for 30 min, agitated with a vortex mixer for 10 s, and centrifuged at $16,000 \times g$ for 10 min (Eppendorf model 5415C; Brinkmann Instruments, Inc., Westbury, NY). Supernatant (0.5 mL) was transferred into a 2-mL vial containing 1 mL deionized H₂O (dilution factor = 3). Samples were processed on a Technicon Autoanalyzer II (Technicon Industrial Systems, Tarrytown, NY) using procedures described by Broderick and Kang (1980). The Autoanalyzer reacted the sample with phenol reagent (0.05 g C₅FeN₆Na₂O · 2H₂O and 11 mL liquefied C₆H₅OH [90% w/v;] dissolved in 1 L deionized H₂O) and hypochlorite reagent (5 g NaOH, 20.07 g Na₂HPO₄, and 50 mL 5.25% NaClO in 1 L of deionized H₂O); absorbance was read at 630 nm.

Culture substrates were analyzed for partial DM and 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.,

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

Procedures for extraction and measurement of CT in substrates were adapted from methods described by Makkar (2003). Each ground SL sample was mixed thoroughly and duplicate 200 mg subsamples were collected. Ten mL of 70% aqueous acetone (vol/vol) was added to each subsample in 25-mL beakers and the mixture was stirred. Subsamples were agitated in an ultrasonic water bath (P250HT, VWR International; Radnor, PA) filled to a depth of 2 cm with deionized H₂O for two 10-min periods. Samples were allowed to stand for a period of 5 min between agitations. The resulting solution was transferred to 15-mL polyethylene tubes and centrifuged at 3,000 x *g* (4°C) for 10 min. The supernatant containing the extracted CT was immediately decanted into clean, sealable containers and frozen (-20°C) until further analysis was attempted.

Ten µL of each CT-extracted sample was transferred into 6 separate 100 x 12 mm glass test tubes with Teflon-lined caps. Each sample was diluted to achieve dilution factors of 100, 200, and 300 by adding 990, 1990, and 2990 µL 70% aqueous acetone, respectively. Conditions of the assay required that final spectrophotometric absorbance at 550 nm be less than 0.6 (Makkar, 2003). Prior to this procedure, the appropriate dilution for our samples was unknown. In retrospect, a dilution factor of 200 produced absorbance readings below the acceptable limit. A 0.5-mL aliquot of diluted-CT extract from each subsample was transferred into 100 x 12 mm glass test tubes with Teflon-lined caps. One test tube with only reagents was used as a blank. Three mL of butanol-HCl (95:5, vol/vol) and 100 µL of ferric reagent (2 g ferric ammonium sulfate and 16.6 mL 10.8 *N* HCl in 83.4 mL deionized H₂O) were added. Each tube was capped tightly and agitated for 30 s with a vortex mixer. Samples were incubated for 60 min in a water

bath at 100°C. Heated samples were allowed to cool for 30 min; heated samples and blanks were then placed into cuvettes (10 x 10 x 45 mm) for absorbance detection.

Sample absorbance at 550 nm was measured using a visible-light spectrophotometer (Spectronic 20 Genesys; Spectronic Instruments, Garforth, Leeds, UK). Absorbance was adjusted to CT concentration, expressed as leucocyanidin equivalents, using the following formula (Makkar, 2003): % CT = $[(\text{Abs}^{550\text{ nm}} - \text{Blank}^{550\text{ nm}}) \times 78.26 \times \text{dilution factor}] \div (\% \text{DM}/100)$. This formula assumed that the effective $E^{1\%, 550\text{ nm}}$ of leucocyanidin was 460.

Protein-binding capacity of CT was estimated by measuring protein-precipitable phenolics in substrates. Substrate samples were mixed thoroughly and duplicate 200 mg subsamples were collected. Ten mL of 50% aqueous methanol (vol/vol) was added to each subsample in a 25-mL beaker and the mixture was stirred; methanol was used to extract CT in this step because acetone reportedly interfered with the protein-binding capacity of CT (Makkar, 2003). Subsamples were agitated in an ultrasonic water bath (P250HT, VWR International; Radnor, PA) filled to a depth of 1 cm with deionized H₂O for 2 × 10-min periods. Samples were allowed to stand for a period of 5 min between agitations. The resulting solution was transferred to 15-mL polyethylene tubes and centrifuged at 3,000 × *g* (4°C) for 10 min. The supernatant containing the extracted CT was decanted into clean, sealable containers and chilled.

Acetate buffer was prepared by adding 11.4 mL of glacial acetic acid to 800 mL deionized H₂O in a 1-L volumetric flask. Solution pH was adjusted to 4.8 using 4 *N* NaOH and the flask was filled to volume with deionized H₂O; 9.86 g NaCl was added and the flask was agitated until solids were dissolved. A 100-μL aliquot of CT extract from each subsample was transferred to clean 15-mL polyethylene centrifuge tubes in duplicate; 0.9 mL 50% methanol and 2 mL bovine serum albumin solution (100 mg bovine serum albumin [fraction V] in 100 mL

acetate buffer) were added to each centrifuge tube to form a tannin-protein complex and the mixture was agitated for 30 s with a vortex mixer. Tubes were chilled for 16 h (4°C) and then centrifuged at $3,000 \times g$ (4°C) for 10 min. Supernatant was discarded and the pellet was dissolved in 1.5 mL of 1% SDS (wt/vol). One mL of each subsample was transferred to a 100×12 mm glass test tube; 3 mL of SDS-triethanolamine solution (1 g SDS and 7 mL triethanolamine [TEA] in 93 mL deionized H₂O) and 1 mL FeCl₃ reagent (0.81 g FeCl₃ in 500 mL 0.1 N HCl) were added. The solution was agitated for 30 s with a vortex mixer and allowed to rest at room temperature for 30 min before transfer to cuvettes (10 x 10 x 45 mm) for absorbance detection.

While subsamples were resting, standards were prepared containing 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 or 0.7 mL tannic acid solution (3 mg tannic acid in 10 mL 1% SDS solution) added to 1 mL FeCl₃ reagent, 3 mL SDS-TEA solution, and enough 1% SDS to bring the total volume to 5 mL in a 100×12 mm glass test tube. Standards were agitated for 30 s with a vortex mixer and transferred to cuvettes (10 x 10 x 45 mm) for absorbance detection. Total phenolics in the original extract were estimated also at this time in order to express protein-precipitable phenolics (i.e., CT that bind protein) as a percentage of total phenolics. A 500- μ L aliquot of each CT subsample (previously extracted from SL with 50% methanol) was added to 0.95 mL 1% SDS, 3 mL SDS-TEA solution, and 1 mL FeCl₃ reagent in a 100×12 mm glass test tube. Samples were agitated for 30 s with a vortex mixer and transferred to cuvettes (10 x 10 x 45 mm) for absorbance detection.

Absorbance of standards, samples of total phenolics, and samples of protein-precipitable phenolics was read at 510 nm using a visible-light spectrophotometer (Spectronic 20 Genesys; Spectronic Instruments, Garforth, Leeds, UK). A linear regression equation was calculated from

absorbance observed for standards to estimate the concentration of tannic-acid equivalents (mg) in samples. Resulting estimates of concentration were multiplied by 1.5. This calculation was necessary because the pellet representing the tannin-protein complex was dissolved in 1.5 mL of 1% SDS. Protein-precipitable phenolics were expressed as a percentage of total phenolics.

Statistical Analysis

In vitro DM disappearance and concentrations of NH₃, total VFA, acetate, propionate, butyrate, valerate, and branched-chain VFA were analyzed using a 2 × 3 × 2 × 3 factorial arrangement of a randomized complete block design (PROC MIXED; SAS Inst. Inc., Cary, NC). Class factors included animal species (*Bos taurus*, *Ovis aries*, or *Capra hircus*), culture substrate (tannin-free or high-CT), microbial suppressant (none, penicillin + streptomycin, or cycloheximide), animal cohort (i.e., block; 1, 2, or 3), animal exposure to tannins (non-exposed or exposed), and length of incubation (4, 8, 12, 24, 36, or 48 h). The model statement included terms for the fixed effects of animal species, culture substrate, microbial suppressant, prior dietary CT exposure, and incubation time, and all possible 2-, 3-, 4-, and 5-way interactions. The random statement had terms for cohort, cohort × species, and prior dietary CT exposure (cohort × species). When protected by a significant F-test ($P < 0.001$), means were separated using the method of Least Significant Difference. Least-squares means for the highest-order, significant ($P < 0.001$) interaction term were reported.

Results and Discussion

In Vitro Dry Matter Disappearance

The effects of culture substrate on *in vitro* DM disappearance (IVDMD) were influenced ($P < 0.001$) by incubation time. Cultures with tannin-free substrate had greater (LSD = 3.69) IVDMD than cultures with the high-CT substrate after 24, 36, and 48 h of incubation (Figure

3.1). *In vitro* DM disappearance in cultures with tannin-free and high-CT substrates generally increased between 4 and 36 h of incubation; however, IVDMD did not change between 36 and 48 h of incubation. The extent of IVDMD after 48 h of incubation was roughly 8.5% greater for cultures with tannin-free substrate than for cultures with high-CT substrate. Getachew et al. (2008) reported that batch cultures fed high-CT substrates had approximately 17% lesser DM disappearance than cultures fed tannin-free substrates.

Ammonia Concentrations

A 4-way interaction effect on ammonia concentration was detected ($P < 0.001$; donor-animal species \times culture substrate \times antimicrobial \times donor-animal tannin-exposure status). Mean ammonia concentrations during a 48-h incubation ranged among treatments between 13.9 and 22.2 mM (LSD = 4.01; Figure 3.2). Across donor-animal species and within antimicrobial treatment, there were small, but consistent differences in ammonia concentrations when tannin-free substrate was used (with or without dietary-CT exposure) or when high-CT growth media was used in conjunction with prior dietary-CT exposure. Among these treatments, suppression of bacterial fermentative activity generally produced the greatest concentrations of ammonia, whereas suppression of fungal fermentative activity or the absence of microbial suppression resulted in slightly lesser ammonia concentrations. The exception to this pattern occurred when ruminal inoculum from *Bos taurus*, *Ovis aries*, and *Capra hircus* was fed high-CT substrate without the benefit of prior CT exposure. In these cases, suppression of fungal fermentative activities resulted in greater ammonia concentrations than suppression of bacterial fermentative activities; antimicrobial-free cultures generally produced intermediate ammonia concentrations. According to Satter and Slyter (1974), ammonia concentrations in *in vitro* systems were the net result of simultaneous amino-acid deamination and ammonia uptake by ruminal microbes. Frutos

et al. (2004) reported a reduction in NH₃-N concentration of about 25% in sheep-, goat-, and deer-inoculum cultures containing CT when compared to cultures not containing CT; there were no changes in cultures with cattle inoculum attributable to CT. We interpreted our results to suggest that, in the absence of prior CT exposure, suppression of the fermentative activities of bacteria in the presence of dietary CT resulted in diminished ammonia production. In contrast, suppression of the fermentative activities of ruminal fungi under the same conditions of diet and exposure resulted in some of the greatest ammonia concentrations observed in our experiment. We speculated that, when fungal fermentation was suppressed, production of ammonia exceeded ammonia uptake. Prior exposure to CT appeared to improve the capabilities of ruminal bacteria to deaminate amino acids in the presence of dietary CT.

Total VFA Concentrations

The effect of substrate type on total VFA concentrations was influenced ($P < 0.001$) by prior exposure to dietary CT and by time. At 4 and 8 h of incubation, there were no differences (LSD = 6.59) in total VFA concentrations between treatments (Figure 3.3). Thereafter, there was a gradual increase in total VFA concentrations in cultures containing tannin-free substrate, regardless of donor animal CT exposure status, with increasing incubation time. Total VFA concentration in tannin-free cultures with inoculum from CT-exposed donors was greater at 36 and 48 h of incubation than that in tannin-free cultures without prior CT exposure. At 12, 24, 36, and 48 h of incubation, cultures with high-CT substrate had lesser total VFA concentrations than cultures with tannin-free substrate. Prior donor-animal exposure to CT had no effect on total VFA concentration when high-CT substrate was used. Lesser total VFA concentration in the presence of substrate CT was reported by several researchers (Waghorn, 2008; Tan et al., 2011;

Hassanat and Benchaar, 2012). Under the conditions of our experiment, it did not appear that prior exposure to CT alleviated the effects of dietary CT on total VFA production.

The effect of substrate type on total VFA concentrations was also influenced ($P < 0.001$) by addition of antimicrobials and by time (Figure 3.4). Tannin-free cultures without antimicrobial additive and tannin-free cultures spiked with cycloheximide (i.e., suppressed fungal fermentative activities) had greater (LSD = 4.37) total VFA concentrations than other treatments at 12, 24, 36, and 48 h of incubation; total VFA concentration under these conditions generally increased with incubation time. Conversely, tannin-free cultures in which bacterial fermentative activities were suppressed with penicillin + streptomycin had less total VFA than corresponding antimicrobial-free or cycloheximide-spiked cultures from 8 to 48 h of incubation. A similar pattern was noted among cultures with high-CT media. High-CT cultures that were antimicrobial-free or that had fungal fermentative activities suppressed had greater total VFA concentrations from 8 to 48 h of incubation than high-CT cultures in which bacterial fermentative activities were suppressed. We interpreted these data to indicate that: 1) dietary CT had strong negative effects on total VFA concentration and 2) bacterial fermentative activities were of greater importance to total VFA concentration than fungal fermentative activities, in the presence or absence of dietary CT. Interestingly, total VFA concentrations at each incubation time point were not different between high-CT cultures without antimicrobial additive and tannin-free cultures in which bacterial fermentative activities were suppressed. Under the conditions of our experiment, dietary CT and suppression of bacterial fermentation may have produced similar degrees of depression in total VFA concentrations.

Acetate Concentrations

The effects of culture substrate on acetate concentrations were influenced ($P < 0.001$) by prior exposure of donor animals to dietary CT. Acetate concentrations in tannin-free cultures with inoculum from non-exposed donors had greater (LSD = 5.64; 36.3 mM) acetate concentrations than high-CT cultures (29.4 and 26.3 mM for non-exposed and CT-exposed inoculum, respectively; Figure 3.5). Acetate concentrations in tannin-free cultures with inoculum from CT-exposed animals (31.1 mM) were intermediate to and not different from other treatments. Tan et al. (2011) and Hassanat and Benchaar (2012) reported that CT did not influence acetate molar proportions *in vitro*; however, absolute concentrations of acetate were not reported in those studies. Under the conditions of our experiment, prior exposure of ruminal microbes to dietary CT did not increase acetate concentrations when high-CT media was used.

Acetate concentrations were also influenced ($P < 0.001$) by inclusion of antimicrobials in culture media and incubation time (Figure 3.6). Acetate concentrations in tannin-free cultures with either no antimicrobial additive or with cycloheximide had greater (LSD = 3.29) acetate concentrations from 12 to 48 h of incubation than other treatments; moreover, acetate concentrations in these treatments were not different from one another at any incubation time point. Tannin-free cultures in which bacterial activity was suppressed had less acetate at 12, 24, 36, and 48 h of incubation than corresponding cultures with no added antimicrobial or those in which fungal activity was suppressed. Cultures with high-CT media followed a similar pattern; however, the effects of incubation time and antimicrobial treatments on acetate concentrations were generally diminished compared to corresponding tannin-free treatments. Shifts in acetate concentration under these circumstances were interpreted to indicate that dietary CT and suppression of bacterial fermentative activities produced similar degrees of depression in acetate concentration. Reductions in acetate concentration in the presence of CT may have been related

to bonding of CT to carbohydrates (Makkar, 2003), whereas reductions of acetate concentrations in the absence of bacterial fermentative activities may have been related to reduced structural carbohydrate digestion (Min et al., 2003; Min et al., 2005).

Propionate Concentration

The effects of culture substrate on propionate concentrations were influenced ($P < 0.001$) by species of the inoculum donor (Figure 3.7). Propionate concentrations were greater (LSD = 0.602) in cultures with tannin-free substrate than in cultures with high-CT substrate across donor-animal species (Figure 3.7). With tannin-free substrate, propionate concentrations were greatest in *Ovis aries* cultures, slightly less in *Bos taurus* cultures, and least in *Capra hircus* cultures (12.3, 11.2, and 10.8 mM, respectively). With high-CT substrate, propionate concentrations were lesser in *Capra hircus* cultures than in cultures with either *Bos taurus* inoculum or *Ovis aries* inoculum (8.2, 9.2, and 9.1 mM, respectively). Frutos et al. (2004) reported that propionate concentrations in cattle-inoculum cultures were reduced by about 60% by CT; however, CT had no effect on propionate in sheep-, goat-, and deer-inoculum cultures. Conversely, Tan et al. (2011) and Hassanat and Benchaar (2012) reported that propionate molar proportions in cattle inoculum did not change in the presence of CT; however these researchers did not indicate if propionate concentrations were altered. *Bos taurus* consumed less supplemental CT than *Ovis aries* or *Capra hircus* during the CT-exposure phase of our experiment (i.e., period 2); therefore, our estimates of propionate concentrations when *Bos taurus* inoculum was fed with high-CT substrate may have been artificially high.

The effects of culture substrate on propionate concentrations were influenced ($P < 0.001$) by antimicrobial treatment and incubation time (Figure 3.8). Cultures with tannin-free growth media that either had no added antimicrobial or had fungal fermentation suppressed with

cycloheximide had greater (LSD = 1.47) propionate concentrations than all other treatments from 8 to 48 h of incubation; there were no differences in propionate concentrations between these treatments at individual time points. Independent of substrate type, cultures in which bacterial fermentative activities were suppressed with penicillin + streptomycin had the least concentrations of propionate. Antimicrobial-free and cycloheximide-spiked cultures with high-CT growth media produced propionate concentrations that were intermediate to and less than those of tannin-free cultures without antimicrobial or with cycloheximide and greater than those of cultures spiked with penicillin + streptomycin. Dietary CT and suppression of bacterial fermentative activities produced similar magnitudes of depression in total VFA concentrations and in acetate concentrations in our study. In contrast, propionate concentrations were more strongly influenced by suppression of bacterial fermentation than by the presence of substrate CT.

Butyrate Concentration

The effects of inoculum-donor species on butyrate concentration were influenced ($P < 0.001$) by antimicrobial addition (Figure 3.9). Across inoculum-donor species, butyrate concentrations were greatest (LSD = 0.222) in antimicrobial-free cultures, intermediate in cycloheximide-spiked cultures, and least in penicillin + streptomycin-spiked cultures. Within antimicrobial treatment, *Bos taurus* inoculum produced the greatest butyrate concentrations and *Capra hircus* inoculum produced the least butyrate concentrations; butyrate concentrations produced by *Ovis aries* inoculum were intermediate to and different from those produced by *Bos taurus* and *Capra hircus* inoculum. Conversely, data published by Frutos et al. (2004) were interpreted to suggest that *in vitro* butyrate concentrations in sheep-inoculum cultures were greater than those of goats and deer, which were greater than those of cattle. Prigge et al. (1984)

reported no differences in butyrate molar proportions between cattle and sheep. Clear species differences in butyrate concentration in our study were accompanied by the observation that antimicrobial additives had similar patterns of influence across inoculum-donor species. We speculated that species differences in butyrate concentrations may have been related to under-consumption of the targeted supplemental dose of CT by *Bos taurus* during period 2 of our study.

Butyrate concentrations were also affected ($P < 0.001$) by culture substrate, antimicrobial additive, and incubation time (Figure 3.10). Tannin-free cultures with no antimicrobial additive and tannin-free cultures spiked with cycloheximide had greater (LSD = 0.44) butyrate concentrations than tannin-free cultures spiked with penicillin + streptomycin at 12, 24, 36, and 48 h of incubation. Cultures with high-CT substrate followed a similar pattern; however, effects of antimicrobial treatments on butyrate concentrations over time were of lesser magnitude than those observed in corresponding tannin-free cultures. Reduction in butyrate molar proportions in the presence of CT were also reported by Getachew et al. (2008) and Hassanat and Benchaar (2012).

Valerate Concentration

The effects of donor-animal species on *in vitro* valerate concentrations were influenced ($P < 0.001$) by antimicrobial additive (Figure 3.11). Across antimicrobial treatments, valerate concentrations were greater (LSD = 0.2221) in cultures containing *Bos taurus* inoculum than in cultures containing *Ovis aries* or *Capra hircus* inoculum. Within antimicrobial treatments and across inoculum-donor species, valerate concentrations were greatest in antimicrobial-free cultures, intermediate in cycloheximide-spiked cultures, and least in penicillin + streptomycin-spiked cultures. It appeared that *Bos taurus* inoculum produced more valerate than *Ovis aries*

and *Capra hircus* under similar fermentation conditions; moreover, the influence of antimicrobial additives appeared uniform across species. In contrast, Prigge et al. (1984) reported no differences in valerate molar proportions between cattle and sheep. We suspect the greater valerate concentrations in *Bos taurus* cultures may have due to incomplete intake of CT-containing supplement compared with *Ovis aries* or *Capra hircus*, which resulted in a lesser degree of ruminal CT-protein complexes to interfere with digestion during period 2 of our study.

Valerate concentrations *in vitro* were also affected ($P < 0.001$) by culture substrate, antimicrobial additive, and incubation time. Tannin-free substrate without antimicrobial and tannin-free substrate with added cycloheximide had greater (LSD = 0.05) valerate concentrations than other treatments at 12, 24, 36, and 48 h of incubation; there were no meaningful differences in valerate concentrations between these treatments at individual time points. Antimicrobial-free and cycloheximide-spiked cultures with high-CT growth media produced valerate concentrations that were less than those of corresponding tannin-free cultures. As with other individual VFA concentrations, the tendency was for cultures treated with penicillin + streptomycin to have the least valerate concentrations. We concluded that dietary CT and our antibacterial treatment (penicillin + streptomycin) had similar influences on valerate concentrations.

Branched-chain VFA Concentration

The effects of culture substrate on *in vitro* branched-chain VFA (BCVFA; isovalerate + isobutyrate) concentrations were influenced ($P < 0.001$) by donor-animal species (Figure 3.13). Within inoculum-donor species, BCVFA concentrations were greater (LSD = 0.042) in cultures with tannin-free substrate than in cultures with high-CT substrate. Independent of substrate type, BCVFA concentrations were at least 0.5 mM greater in *Bos taurus* cultures than in *Ovis aries* or *Capra hircus* cultures. *Capra hircus* cultures produced slightly more BCVFA than *Ovis aries*

cultures with the tannin-free substrate (0.74 vs. 0.68 mM) but not with the high-CT substrate (0.55 vs. 0.51 mM). Hassanat and Benchaar (2012) reported that at low doses of CT (20 and 50 g CT/kg substrate) *in vitro* BCVFA molar proportions were greater than when CT was excluded. Under the conditions of our study, the presence of CT in culture substrate appeared to influence BCVFA production to the same degree across inoculum-donor species.

As with inoculum-donor species effects on butyrate and valerate in our study, it appeared that *Bos taurus* inoculum produced more BCVFA than either *Ovis aries* or *Capra hircus*. This observation may have been related to the fact that *Ovis aries* and *Capra hircus* in our study consumed the targeted dose of supplemental CT, whereas *Bos taurus* consumed only 67% of the targeted dose. More evidence for this possibility was developed when the influences of donor-species inoculum on BCVFA concentrations were examined over time (Figure 3.14). *Bos taurus* had BCVFA concentrations that were roughly twice ($P < 0.001$) that of *Ovis aries* or *Capra hircus* at each incubation-time endpoint; however, there were no differences in BCVFA concentrations between *Ovis aries* and *Capra hircus* at any incubation-time end point. Prigge et al. (1984) reported no differences in isobutyrate and isovalerate molar proportions between cattle and sheep fed forage diets.

A three-way interaction, independent of inoculum-donor species, was detected ($P < 0.0001$) on BCVFA concentrations that involved culture substrate, antimicrobial additive, and incubation time (Figure 3.15). Tannin-free cultures with no antimicrobial additive and tannin-free cultures spiked with cycloheximide had no increase (LSD = 0.072) in BCVFA concentrations from 4 through 12 h of incubation; thereafter, BCVFA concentrations increased modestly. Conversely, cultures containing tannin-free substrate with penicillin + streptomycin had greater BCVFA concentration with each increase in incubation time. Concentrations of

BCVFA in cultures containing high-CT substrate, regardless of antimicrobial additive, did not change over time. Response in BCVFA concentrations to antimicrobial treatments was different from that of other VFA in our study. Suppression of bacterial fermentative activities appeared to have comparatively little influence on BCVFA concentrations, whereas acetate, propionate, butyrate, and valerate concentrations were decreased dramatically by bacterial suppression. We speculated that fungal fermentative activities may have been relatively more important to BCVFA concentrations than bacterial fermentative activities; moreover, the presence of substrate CT had strong suppressive effects on BCVFA.

Under the condition of our study, the presence of CT reduced IVDMD and total and individual VFA concentrations. It appeared that prior dietary exposure to CT did not negate the negative effects of CT on IVDMD and VFA concentrations. In contrast, prior donor-animal exposure to dietary CT appeared to decrease ammonia uptake or improve the amino acid deamination capabilities of ruminal bacteria in the presence of dietary CT. When bacterial fermentation was suppressed with penicillin + streptomycin, total and individual VFA concentrations were reduced compared to antimicrobial-free and cycloheximide-spiked (i.e., fungal activity suppression) cultures. We interpreted these data to indicate that bacterial fermentative activities were of greater importance to concentrations of individual VFA than fungal fermentative activities, in the presence or absence of dietary CT. Shifts in total VFA, acetate, butyrate, and valerate were interpreted to indicate that dietary CT and suppression of bacterial fermentative activities produced similar degrees of depression in concentration. Conversely, propionate concentrations were more strongly influenced by suppression of bacterial fermentation than by the presence of substrate CT, whereas suppression of bacterial fermentative activities appeared to have comparatively little influence on BCVFA concentrations.

There were slight differences between inoculum-donor species in propionate, butyrate, valerate, and BCVFA concentrations. With tannin-free substrate, propionate concentrations were greatest in *Ovis aries* cultures, slightly less in *Capra hircus* cultures, and least in *Bos taurus* cultures. Depression in propionate concentration was similar across all species in the presence of CT. It appeared that *Bos taurus* inoculum produced more butyrate, valerate, and BCVFA than *Ovis aries* and *Capra hircus* under similar fermentation conditions; however, intake of CT-containing supplement during period 2 of our study was less by *Bos taurus* (% BW) than that by *Ovis aries* or *Capra hircus*. This may have allowed greater aggregate yields of these VFA under conditions when interaction terms involving inoculum-donor species were significant. The influence of antimicrobial additives appeared to be uniform across inoculum-donor species. Further research on the ability of various ruminant species to dietary CT adaptation appears warranted.

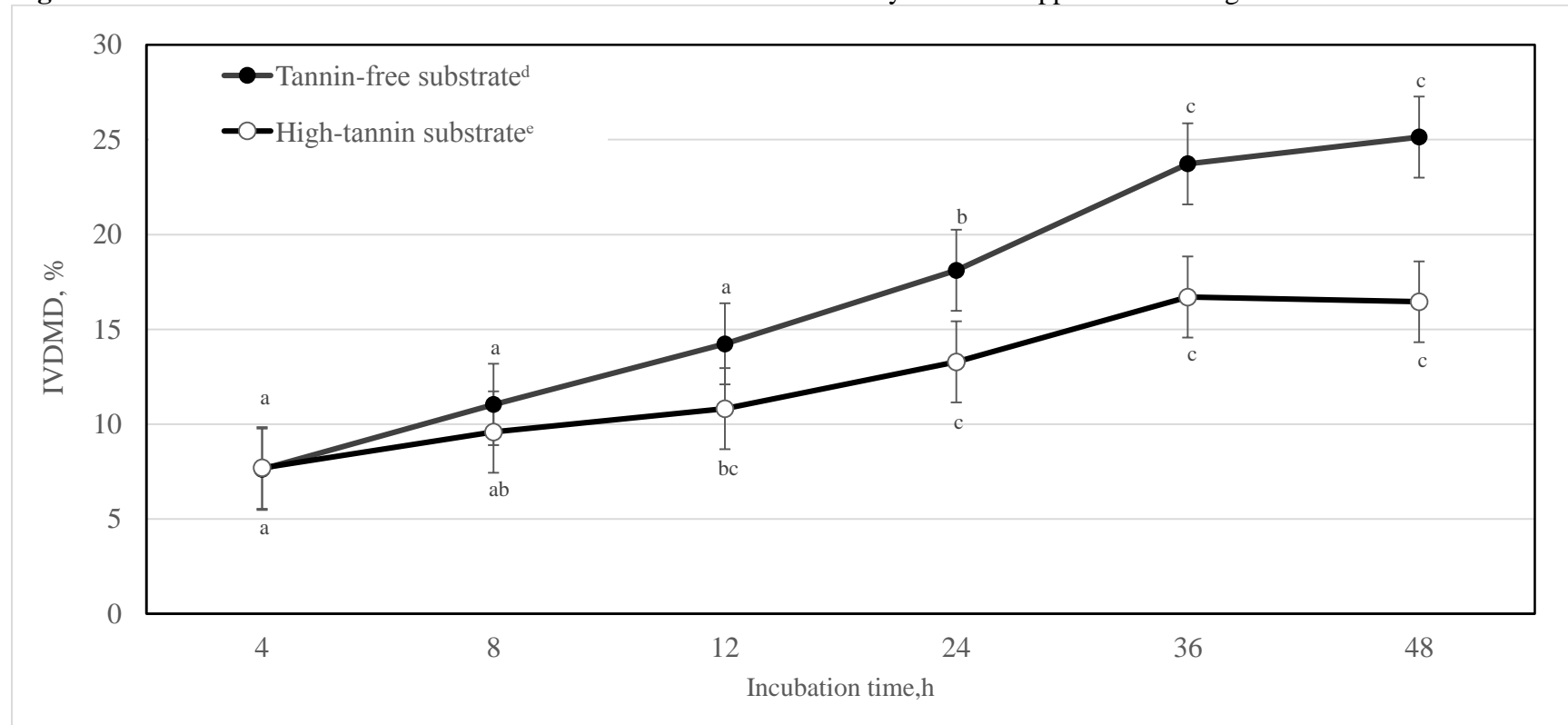
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Figure 3.1. Effects of fermentation time and culture substrate on *in vitro* dry matter disappearance during a 48-h incubation

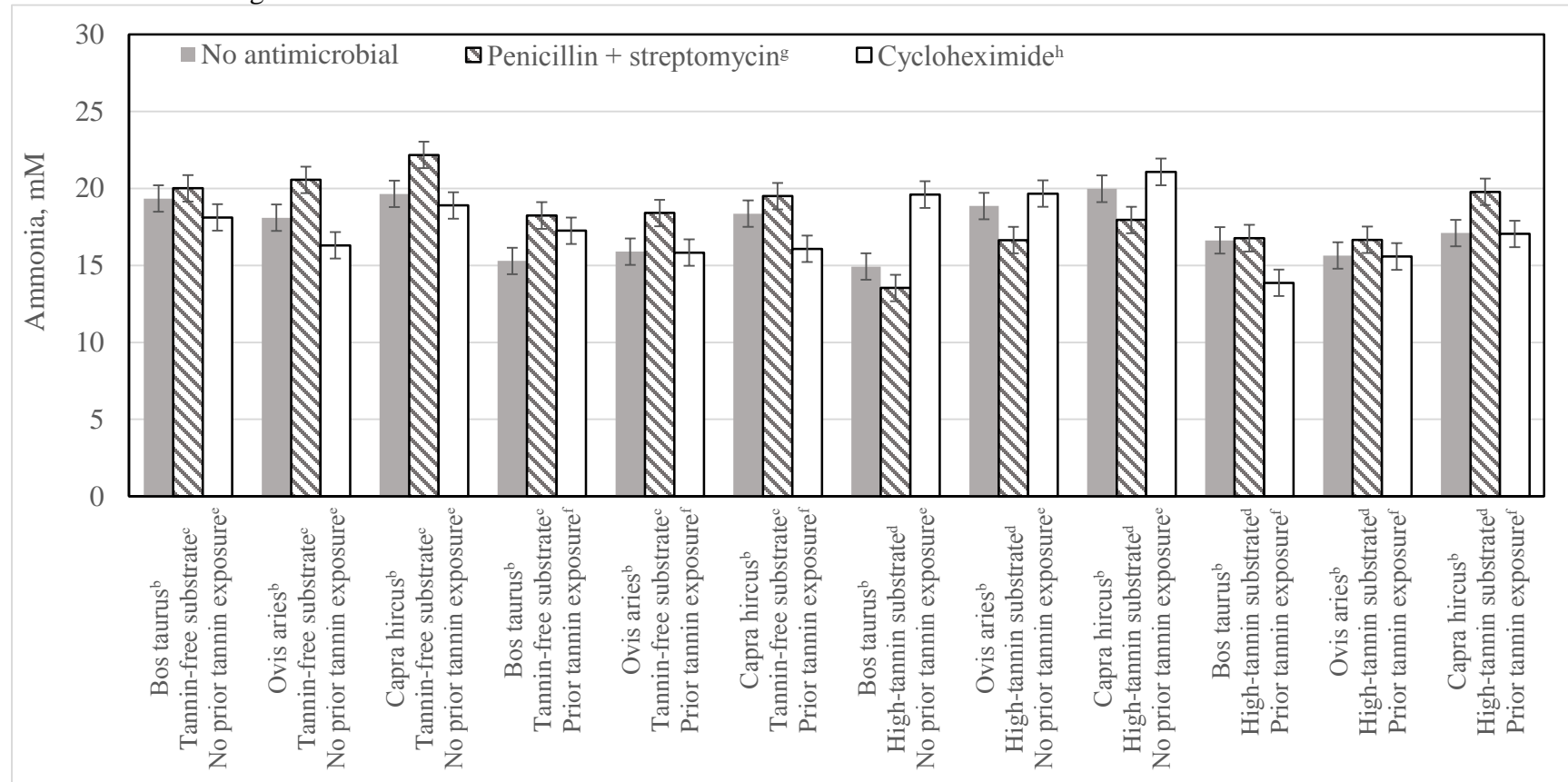


^{a, b, c} Within culture substrate, incubation-time points with unlike superscripts differ ($P < 0.0001$; F-test protected LSD = 3.69). Within incubation-time point, separation of error bars indicates difference between substrate types.

^d Culture substrate consisted of ground smooth bromegrass hay only.

^e Culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

Figure 3.2. Effects of culture substrate, donor-species inoculum, antimicrobial, and prior dietary tannin exposure on mean ammonia concentrations during a 48-h *in vitro* incubation^a



^a Culture substrate × donor-species inoculum × antimicrobial × prior dietary tannin exposure ($P = 0.0002$; F-test protected LSD = 3.09).

^b $n = 3/\text{species}$.

^c Culture substrate consisted of ground smooth bromegrass hay only.

^d Culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

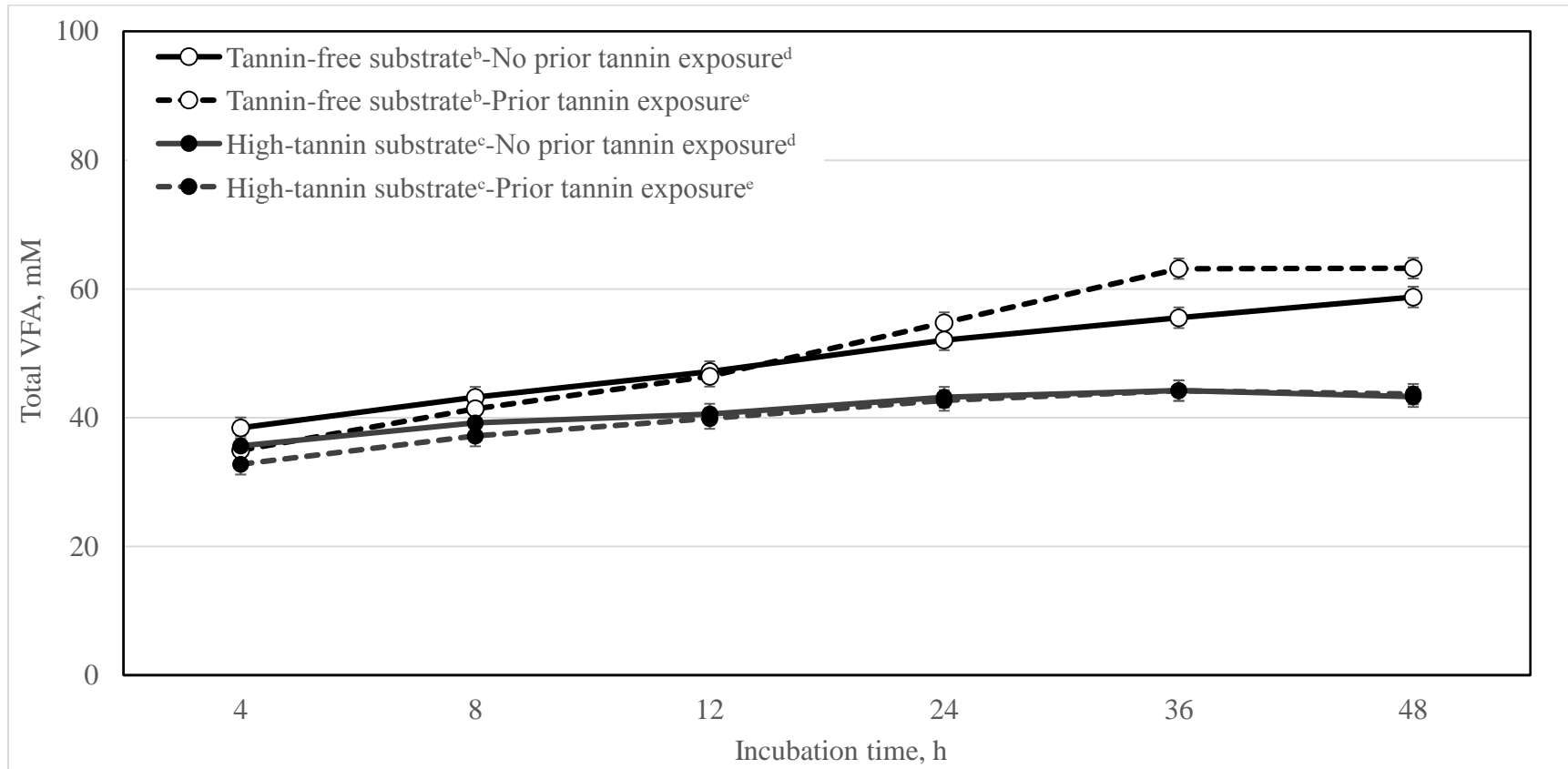
^e Cultures contained ruminal fluid collected from animals fed a tannin-free diet.

^f Cultures contained ruminal fluid collected from animals adapted to a high-tannin diet for 21d.

^g Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^h Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Figure 3.3. Effects of fermentation time, culture substrate, and prior dietary tannin exposure on total VFA concentrations during a 48-h *in vitro* incubation^a



^aTime × culture substrate × prior dietary tannin exposure ($P < 0.0001$; F-test protected LSD = 3.69).

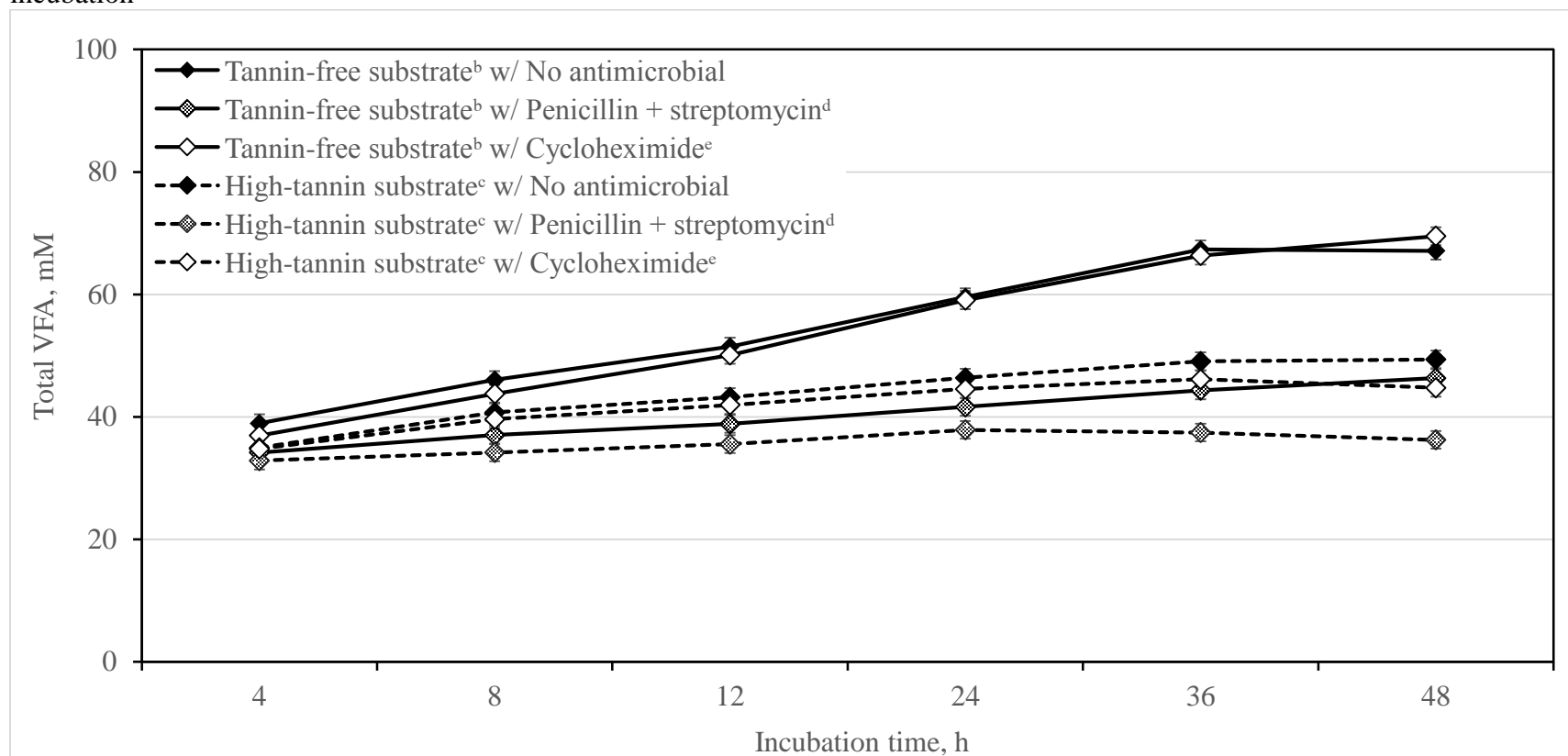
^bTannin-free culture substrate consisted of ground smooth bromegrass hay only.

^cHigh-tannin culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^dCultures contained ruminal fluid collected from animals fed a tannin-free diet.

^eCultures contained ruminal fluid collected from animals fed a high-tannin diet.

Figure 3.4. Effects of fermentation time, culture substrate, and antimicrobial on total VFA concentrations during a 48-h *in vitro* incubation^a



^a Time × culture substrate × antimicrobial ($P < 0.0001$; F-test protected LSD = 0.072).

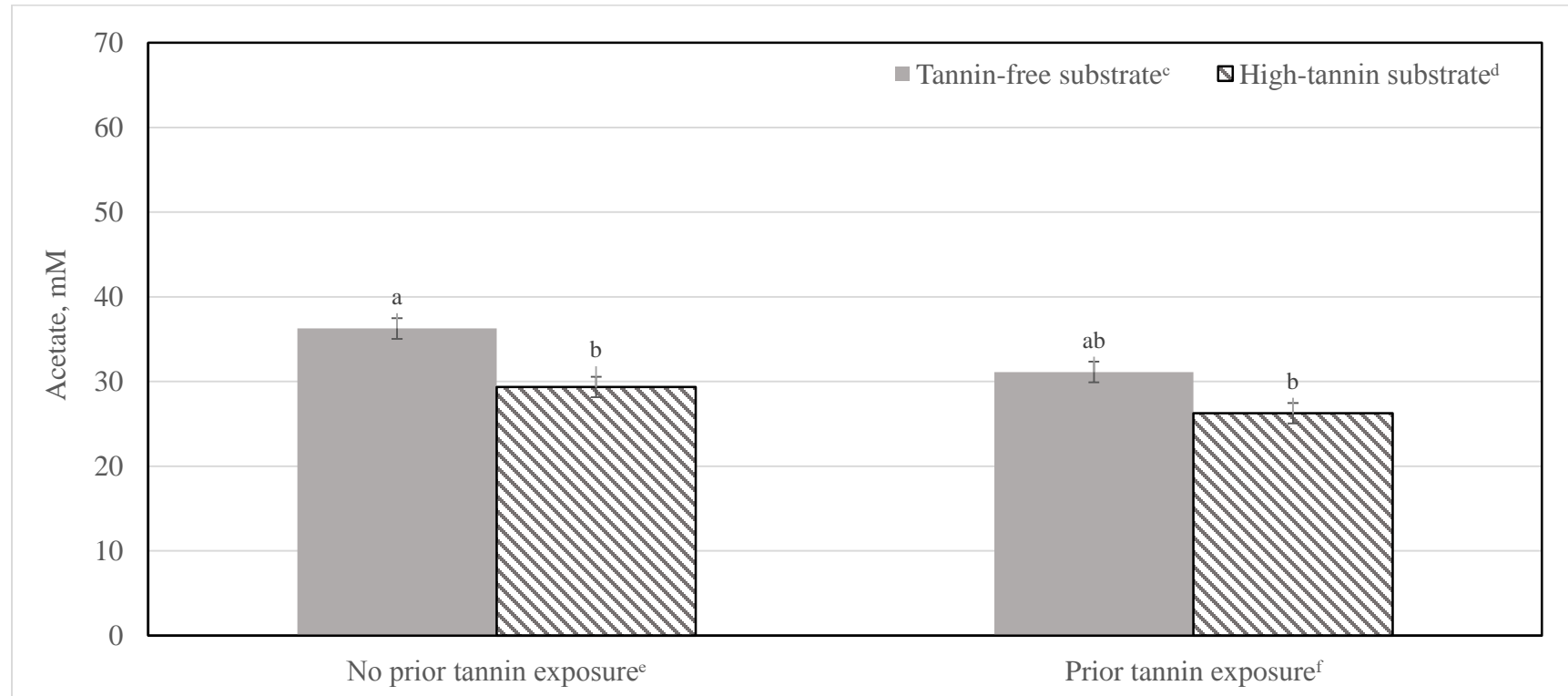
^b Tannin-free culture substrate consisted of ground smooth bromegrass hay only.

^c High-tannin culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^d Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^e Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Figure 3.5. Effects of culture substrate and prior dietary tannin exposure on mean acetate concentrations during a 48-h *in vitro* incubation



^{a, b} Means with unlike superscripts differ ($P < 0.0001$; F-test protected LSD = 5.64).

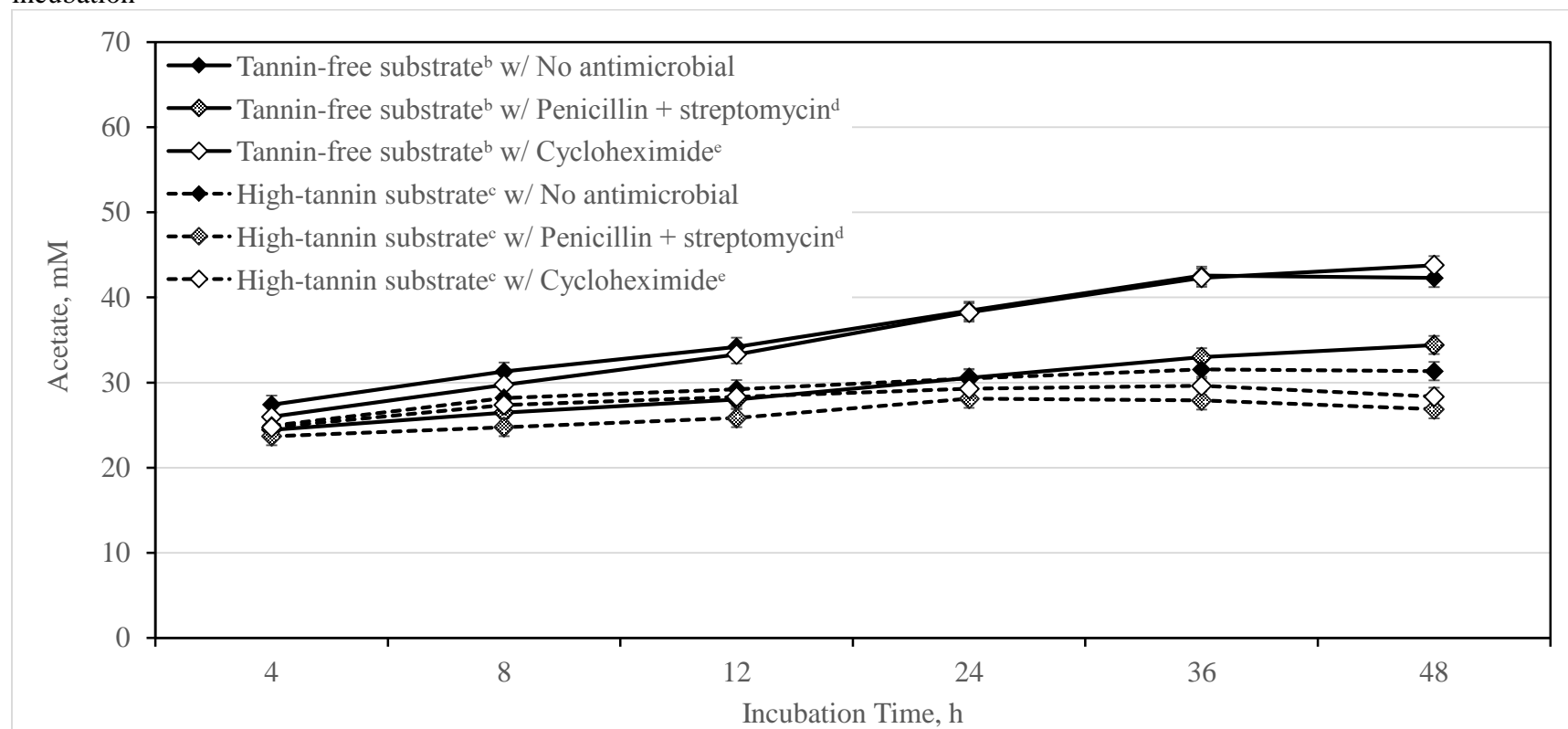
^c Tannin-free culture substrate consisted of ground smooth bromegrass hay only.

^d High-tannin culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^e Cultures contained ruminal fluid collected from animals fed a tannin-free diet.

^f Cultures contained ruminal fluid collected from animals adapted to a high-tannin diet for 21 d.

Figure 3.6. Effects of fermentation time, culture substrate, and antimicrobial on acetate concentrations during a 48-h *in vitro* incubation^a



^a Time × culture substrate × antimicrobial ($P < 0.0001$; F-test protected LSD = 3.29).

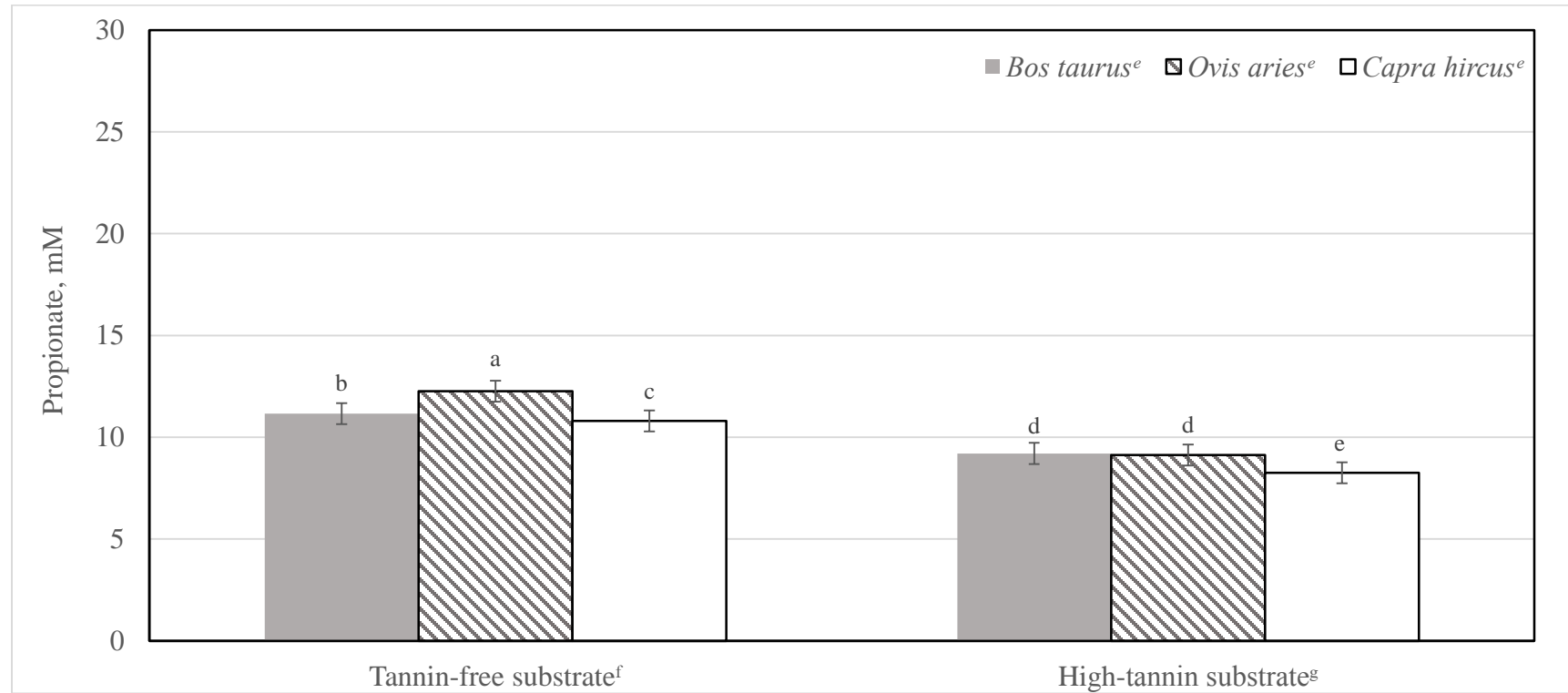
^b Tannin-free culture substrate consisted of ground smooth bromegrass hay only.

^c High-tannin culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^d Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^e Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Figure 3.7. Effects of culture substrate and donor-species inoculum on mean propionate concentrations during a 48-h *in vitro* incubation



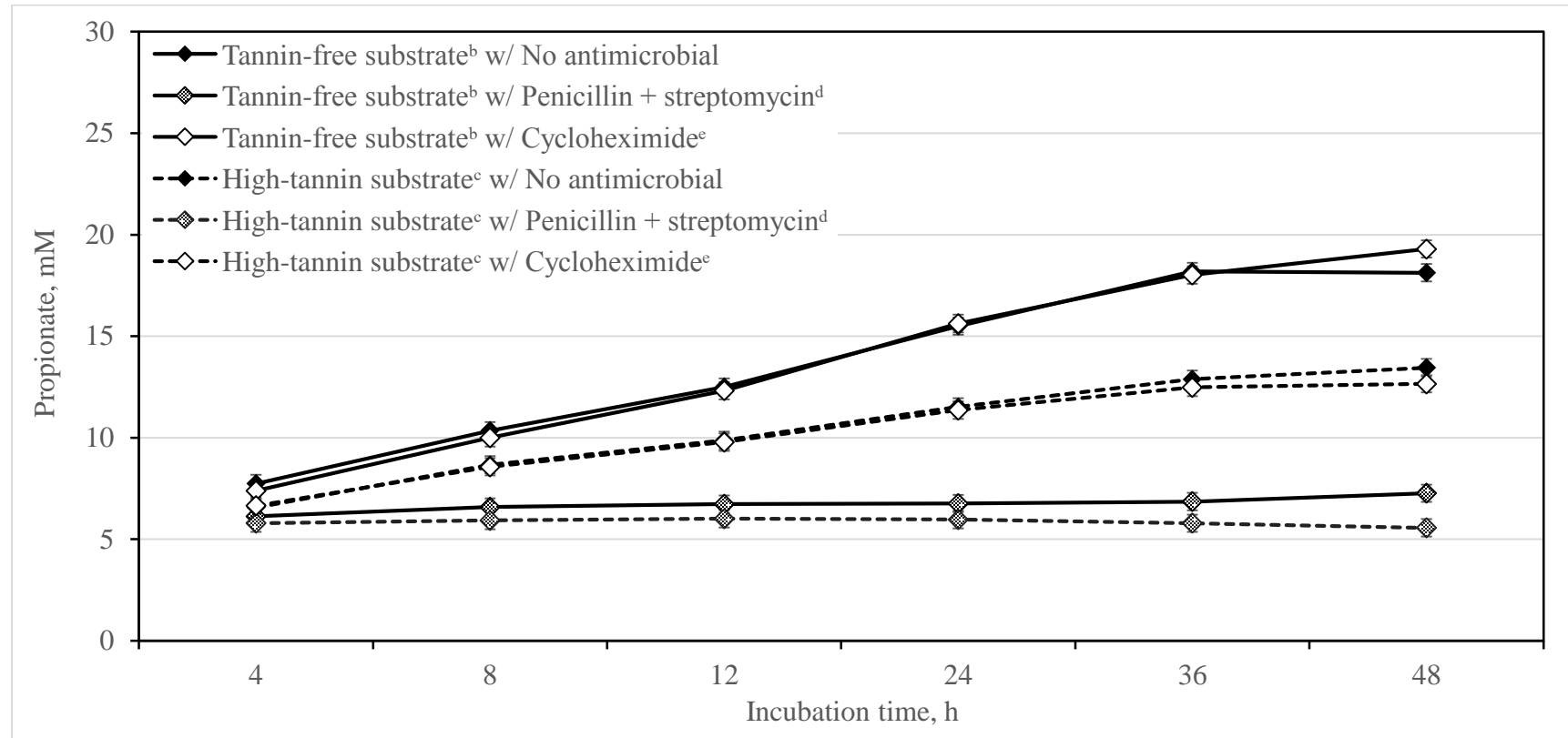
^{a, b, c, d} Means with unlike superscripts differ ($P < 0.0001$; F-test protected LSD = 0.602).

^e n = 3/species.

^f Tannin-free culture substrate consisted of ground smooth bromegrass hay only.

^g High-tannin culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

Figure 3.8. Effects of fermentation time, culture substrate, and antimicrobial on propionate concentrations during a 48-h *in vitro* incubation^a



^a Time \times culture substrate \times antimicrobial ($P < 0.0001$; F-test protected LSD = 0.072).

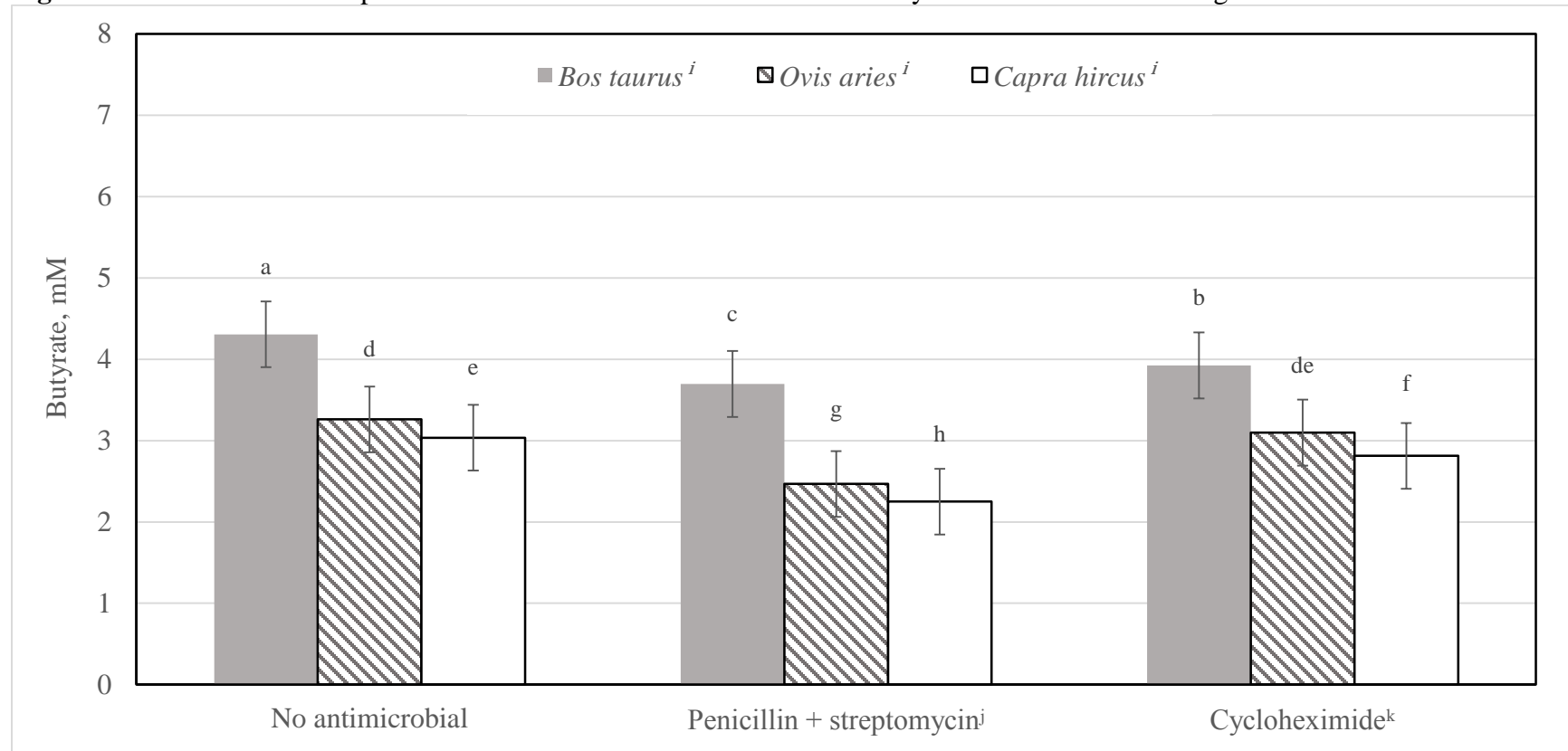
^b Tannin-free culture substrate consisted of ground smooth bromegrass hay only.

^c High-tannin culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^d Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^e Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Figure 3.9. Effects of donor-species inoculum and antimicrobial on mean butyrate concentrations during a 48-h *in vitro* incubation



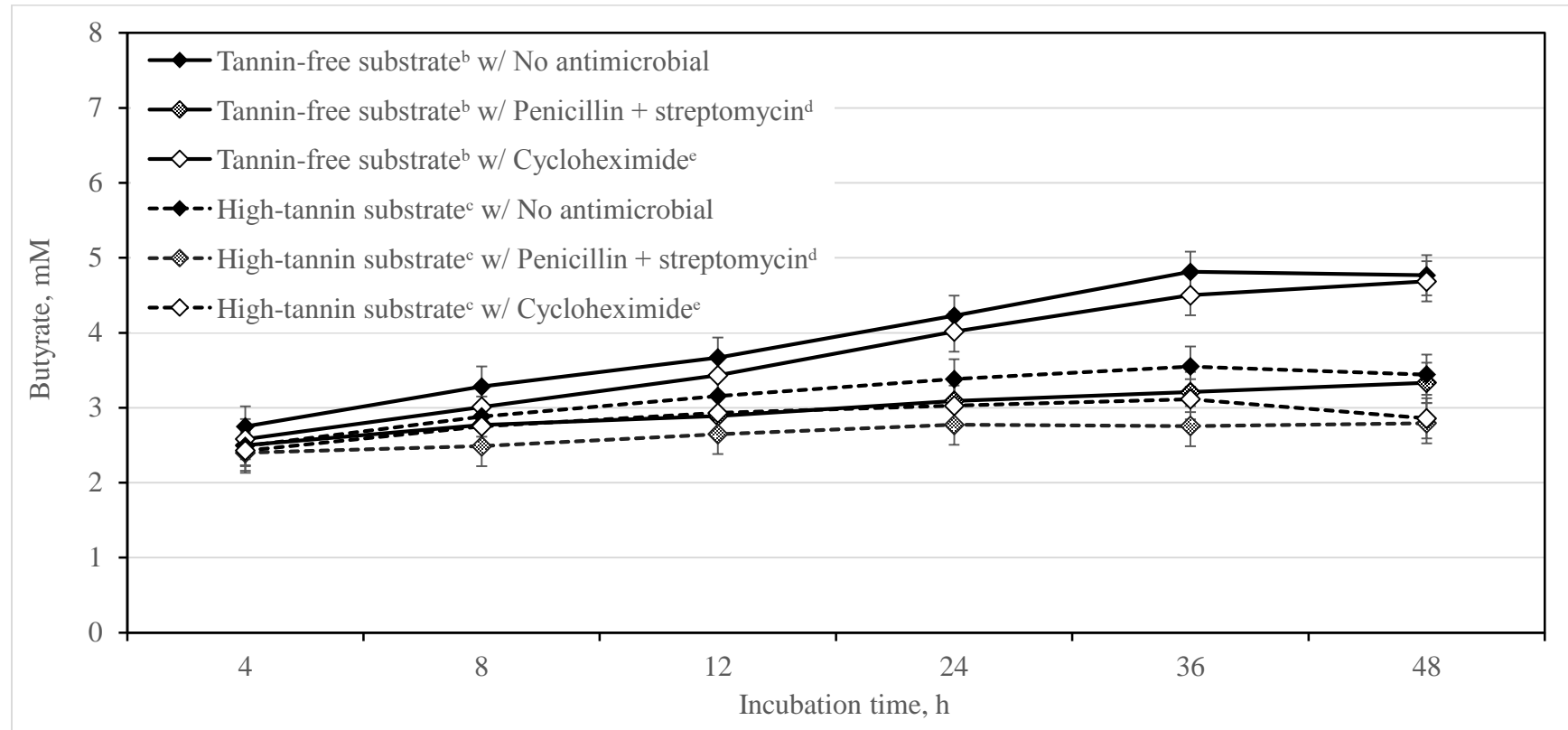
a, b, c, d, e, f, g, h Means with unlike superscripts differ ($P = 0.0005$; F-test protected LSD = 0.22).

ⁱ n = 3/species.

^j Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^k Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Figure 3.10. Effects of fermentation time, culture substrate, and antimicrobial on butyrate concentrations during a 48-h *in vitro* incubation^a



^a Time × culture substrate × antimicrobial ($P < 0.0001$; F-test protected LSD = 0.072).

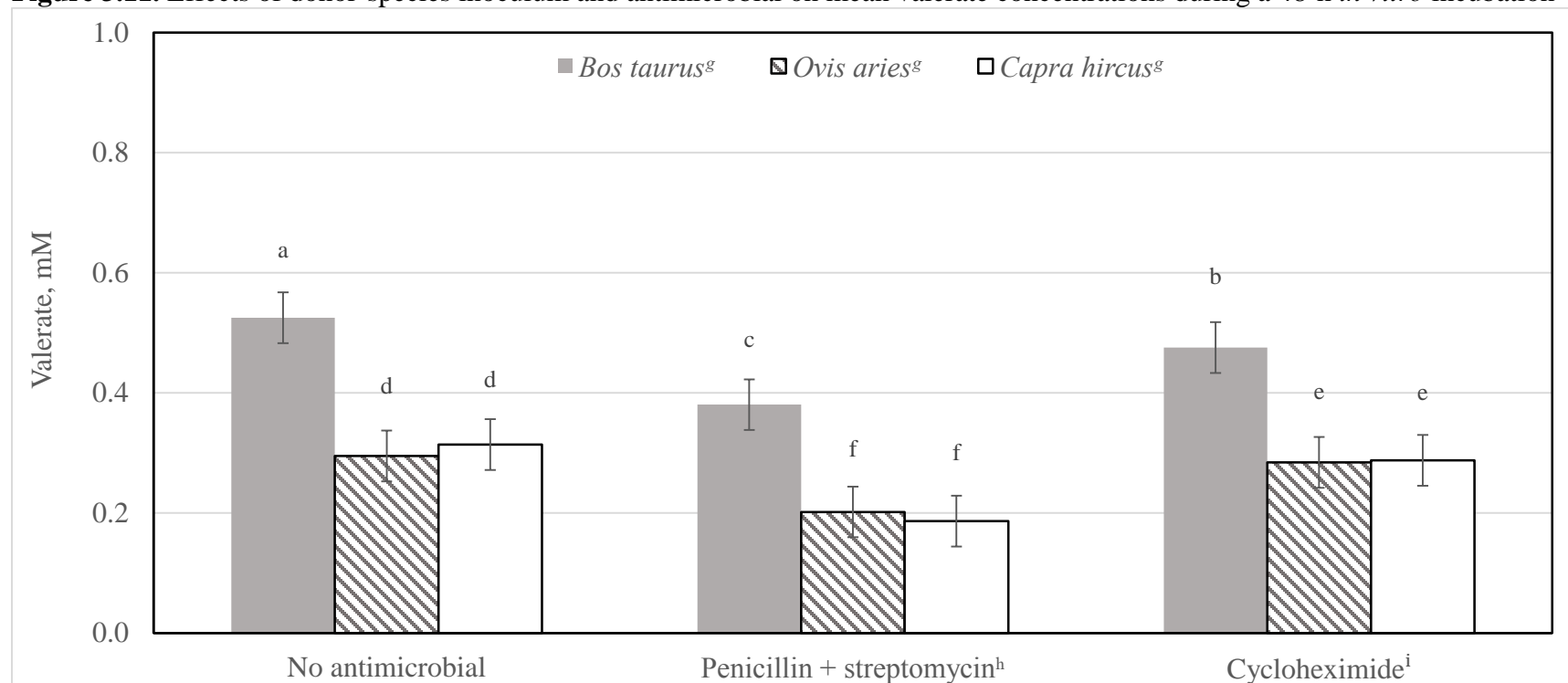
^b Tannin-free culture substrate consisted of ground smooth bromegrass hay only.

^c High-tannin culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^d Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^e Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Figure 3.11. Effects of donor-species inoculum and antimicrobial on mean valerate concentrations during a 48-h *in vitro* incubation



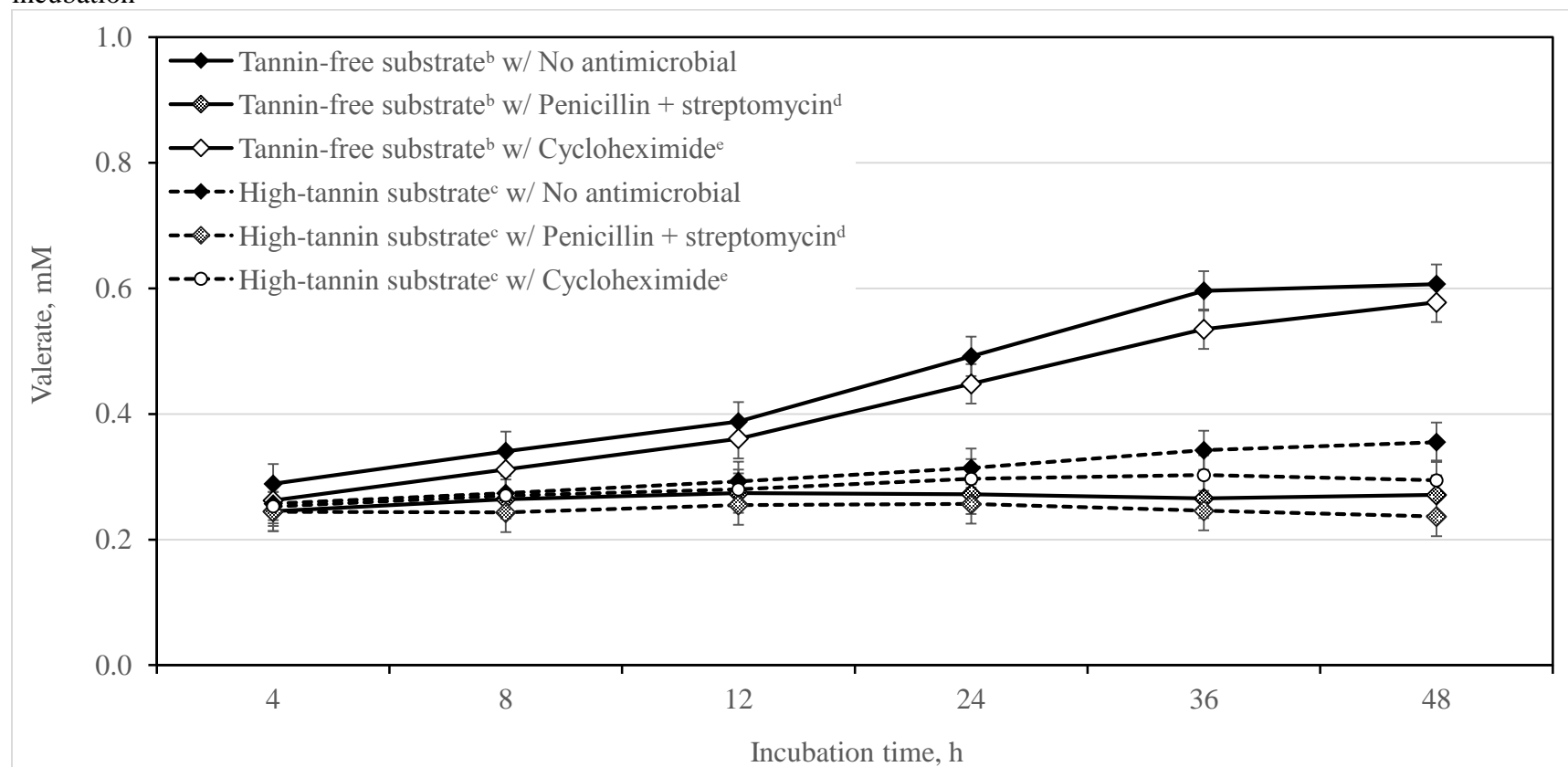
^{a, b, c, d, e, f} Means with unlike superscripts differ ($P < 0.0001$; F-test protected LSD = 0.026).

^g $n = 3/\text{species}$.

^h Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

ⁱ Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Figure 3.12. Effect of fermentation time, culture substrate, and antimicrobial on valerate concentrations during a 48-h *in vitro* incubation^a



^a Time \times culture substrate \times antimicrobial ($P < 0.0001$; F-test protected LSD = 0.072).

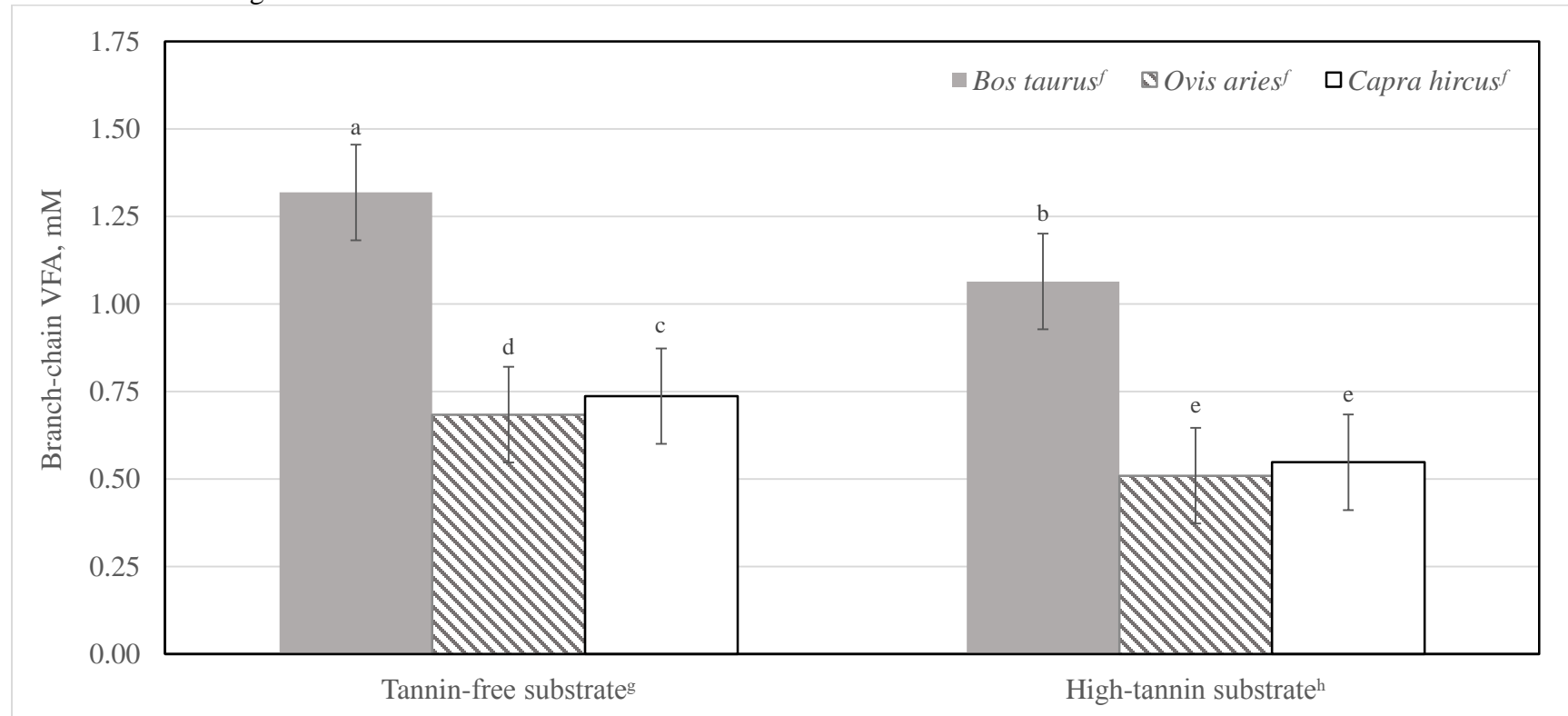
^b Tannin-free culture substrate consisted of ground smooth bromegrass hay only.

^c High-tannin culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^d Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^e Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Figure 3.13. Effects of culture substrate and donor-species inoculum on mean branched-chain VFA (isobutyrate + isovalerate) concentrations during a 48-h *in vitro* incubation



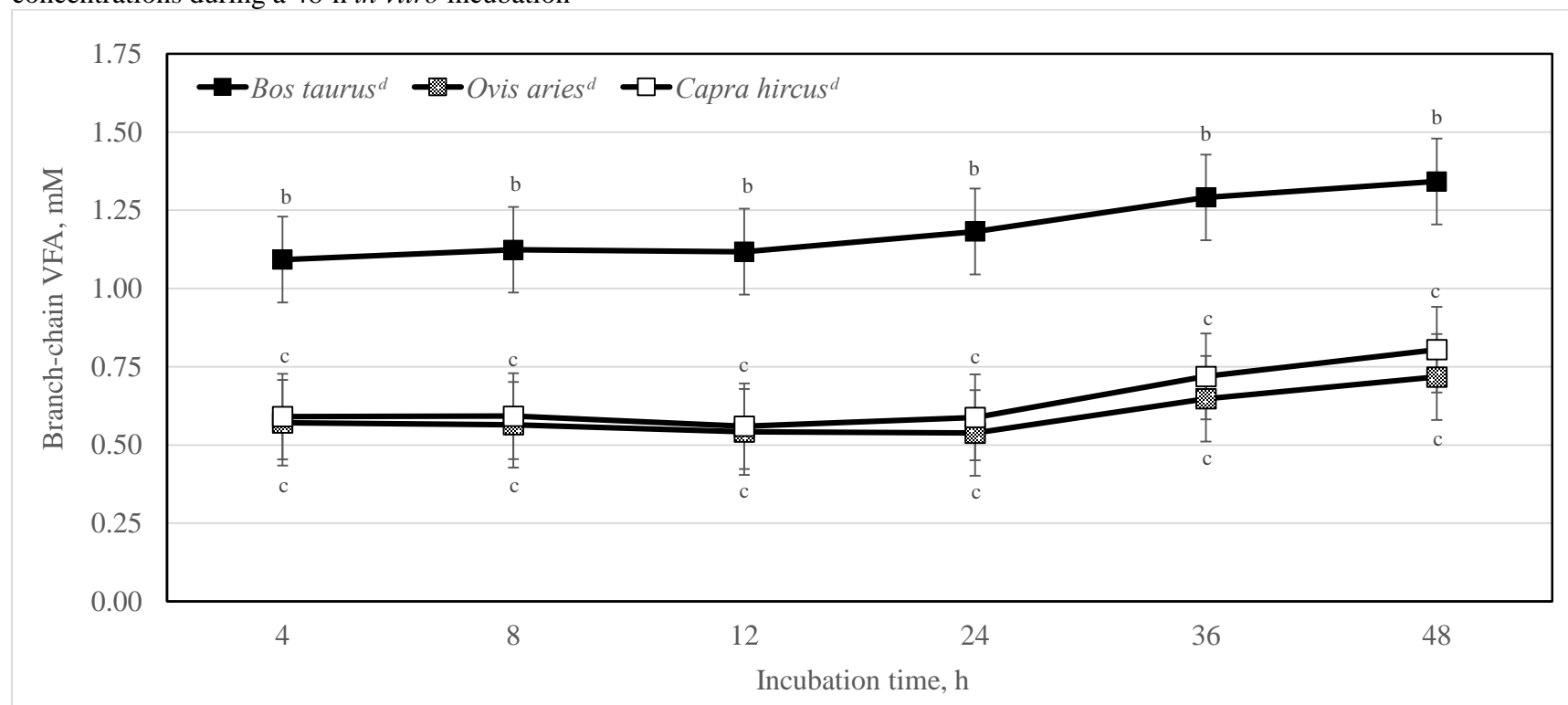
^{a, b, c, d, e} Means with unlike superscripts differ ($P < 0.0001$; F-test protected LSD = 0.042).

^f n = 3/species.

^g Tannin-free culture substrate consisted of ground smooth bromegrass hay only.

^h High-tannin culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

Figure 3.14. Effects of fermentation time and donor-species inoculum on branched-chain VFA (isobutyrate + isovalerate) concentrations during a 48-h *in vitro* incubation^a

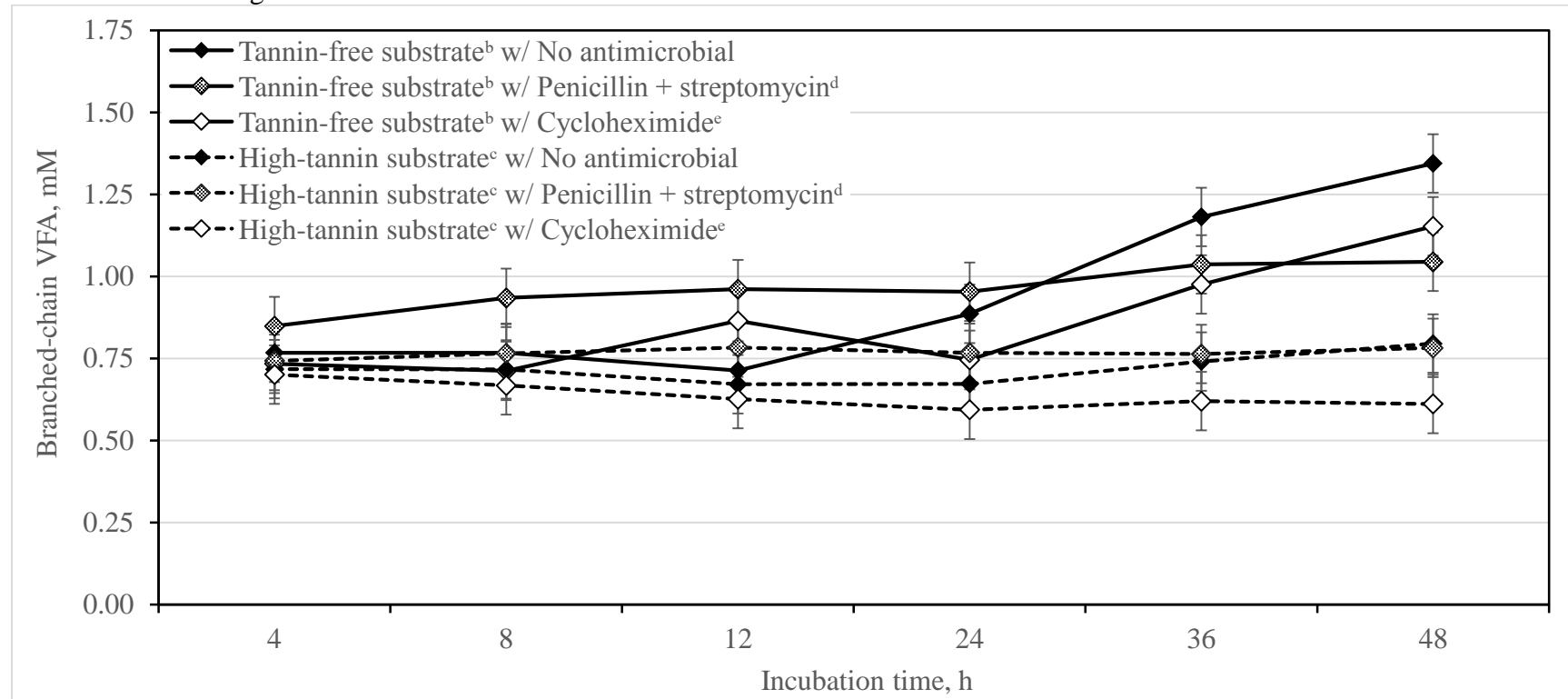


^a Time × donor-species inoculum ($P = 0.001$).

^{b, c} Within incubation time point, means with unlike superscripts differ ($P = 0.001$; F-test protected LSD = 0.605).

^d $n = 3$ /species.

Figure 3.15. Effects of fermentation time, culture substrate, and antimicrobial on branched-chain VFA (isobutyrate + isovalerate) concentrations during a 48-h *in vitro* incubation^a



^aTime × culture substrate × antimicrobial ($P < 0.0001$; F-test protected LSD = 0.072).

^bTannin-free culture substrate consisted of ground smooth bromegrass hay only.

^cHigh-tannin culture substrate consisted of 89.8% ground smooth bromegrass hay and 10.2% condensed tannins of plant origin.

^dCultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^eCultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

Appendix A - *In Vitro* Batch Cultures Tables

Table A.1 Effects of antimicrobial following a 48-h *in vitro* incubation.

Antimicrobial agent	Total gas pressure (psi)	NH ₃ (mM)	Total VFA (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	Valerate (mM)	BCVFA ^f (mM)
No antimicrobial	12.96 ^a	18.96 ^a	79.84 ^a	38.84 ^a	22.22 ^a	5.76 ^a	0.62 ^a	1.16 ^a
Penicillin + streptomycin ^d	21.70 ^b	20.17 ^b	49.45 ^b	48.35 ^b	6.25 ^b	3.24 ^b	0.23 ^b	0.89 ^b
Cycloheximide ^e	22.44 ^b	16.78 ^c	77.12 ^a	50.09 ^b	22.05 ^a	5.22 ^c	0.58 ^a	0.92 ^b

^{a, b, c} Within a column, means with different superscripts differ ($P < 0.001$; F-test protected LSD: 2.38 for total gas production, 3.16 for total VFA concentration, 2.09 for acetate concentration, 1.41 for propionate concentration, 0.29 for butyrate concentration, 0.11 for valerate concentration, and 0.10 for BCVFA).

^d 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^e 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^f BCVFA means branched-chain volatile fatty acids (isobutyrate and isovalerate).

Table A.2 Effects of culture substrate and antimicrobial following a 48-h *in vitro* incubation.

Culture substrate	Antimicrobial agent	Total gas pressure (psi)	NH ₃ (mM)	Total VFA (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	Valerate (mM)	BCVFA ^j (mM)
Tannin-free ^f	No antimicrobial	30.57 ^c	20.44	96.10 ^c	60.46 ^c	26.21 ^c	7.08 ^d	0.79 ^c	1.55 ^c
	Penicillin + streptomycin ^h	16.58 ^{ad}	21.07	57.24 ^d	45.64 ^d	6.72 ^b	3.60 ^c	0.22 ^b	1.05 ^d
	Cycloheximide ⁱ	28.66 ^c	17.46	91.57 ^e	57.51 ^e	25.61 ^c	6.47 ^e	0.75 ^c	1.24 ^e
High-tannin ^g	No antimicrobial	14.30 ^a	17.48	63.59 ^a	39.71 ^a	18.24 ^a	4.44 ^a	0.44 ^a	0.76 ^a
	Penicillin + streptomycin ^h	9.35 ^b	19.28	41.66 ^b	32.03 ^b	5.77 ^b	2.89 ^b	0.24 ^b	0.73 ^{ab}
	Cycloheximide ⁱ	14.75 ^a	16.06	62.67 ^a	39.19 ^a	18.48 ^a	3.98 ^c	0.42 ^a	0.60 ^b

^{a, b, c, d, e} Within a column, means with different superscripts differ ($P < 0.001$; F-test protected LSD: 3.4 for total gas production, 3.43 for total VFA concentration, 2.96 for acetate concentration, 3.41 for propionate concentration, 0.39 for butyrate concentration, 0.16 for valerate concentration, and 0.15 for BCVFA).

^f Culture substrate consisted only of ground bromegrass hay, no quebracho tannin was present.

^g Culture substrate was 10.2% condensed tannins in the form of quebracho tannin and 89.8% ground bromegrass hay.

^h Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

ⁱ Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^j BCVFA means branched-chain volatile fatty acids (isobutyrate and isovalerate).

Table A.3 Effects of culture substrate and prior dietary tannin exposure following a 48-h *in vitro* incubation.

Culture substrate	Prior tannin exposure	Total gas pressure (psi)	NH ₃ (mM)	Total VFA (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	Valerate (mM)	BCVFA ^h (mM)
Tannin-free ^d	No ^f	26.58 ^b	19.41 ^c	83.68 ^c	57.82 ^b	18.93 ^{bc}	5.43	0.52	1.08
	Yes ^g	23.97 ^b	19.90 ^{ac}	79.59 ^d	51.25 ^c	20.19 ^c	6.01	0.65	1.48
High-tannin ^e	No ^f	11.89 ^a	18.54 ^{abc}	52.57 ^a	36.70 ^a	11.68 ^a	3.35	0.33	0.51
	Yes ^g	13.71 ^a	16.67 ^b	59.37 ^b	37.26 ^a	16.65 ^b	4.19	0.40	0.88

^{a, b, c} Within a column, means with different superscripts differ ($P < 0.001$; F-test protected LSD: 3.09 for total gas production, 2.05 for NH₃ concentration, 3.43 for total VFA concentration, 5.89 for acetate concentration, and 3.41 for propionate concentration).

^d Culture substrate consisted only of ground bromegrass hay, no quebracho tannin was present.

^e Culture substrate was 10.2% condensed tannins in the form of quebracho tannin and 89.8% ground bromegrass hay.

^f Cultures contained ruminal fluid collected from animals not fed quebracho tannin supplement.

^g Cultures contained ruminal fluid collected from animals fed quebracho tannin supplement.

^h BCVFA means branched-chain volatile fatty acids (isobutyrate and isovalerate).

Table A.4 Effects of donor-species inoculum and culture substrate following a 48-h *in vitro* incubation.

Animal species	Culture substrate	Total gas pressure (psi)	NH ₃ (mM)	Total VFA (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	Valerate (mM)	BCVFA ^g (mM)
<i>Bos taurus</i> ^d	Tannin-free ^e	27.77	20.48	86.10	57.06 ^b	20.22	6.49	0.76	1.57
	High-tannin ^f	13.21	17.06	57.03	36.78 ^a	14.69	4.22	0.44	0.89
<i>Ovis aries</i> ^d	Tannin-free ^e	23.08	18.56	77.31	51.56 ^c	19.06	5.17	0.47	1.05
	High-tannin ^f	11.41	17.22	53.83	35.77 ^a	14.02	3.22	0.27	0.55
<i>Capra hircus</i> ^d	Tannin-free ^e	24.98	19.92	81.49	54.99 ^b	19.25	5.50	0.54	1.22
	High-tannin ^f	13.78	18.53	57.06	38.38 ^a	13.78	3.87	0.38	0.65

^{a, b, c} Within a column, means with different superscripts differ ($P < 0.001$; F-test protected LSD: 2.96 for acetate concentration).

^d $n = 3$ /species.

^e Culture substrate consisted only of ground bromegrass hay, no quebracho tannin was present.

^f Culture substrate was 10.2% condensed tannins in the form of quebracho tannin and 89.8% ground bromegrass hay.

^g BCVFA means branched-chain volatile fatty acids (isobutyrate and isovalerate).

Table A.5 Effects of antimicrobial and prior dietary tannin exposure following a 48-h *in vitro* incubation.

Antimicrobial agent	Prior tannin exposure	Total gas pressure (psi)	NH ₃ (mM)	Total VFA (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	Valerate (mM)	BCVFA ^h (mM)
No antimicrobial	No ^f	22.89	19.35	78.19	51.34	20.04 ^a	5.32	0.56	0.94
	Yes ^g	21.99	18.56	81.49	48.84	24.41 ^b	6.20	0.68	1.37
Penicillin + streptomycin ^d	No ^f	13.79	20.21	50.83	41.06	5.78 ^c	3.06	0.21	0.72
	Yes ^g	12.14	20.14	48.07	36.61	6.71 ^c	3.43	0.25	1.06
Cycloheximide ^e	No ^f	21.01	17.37	75.37	49.38	19.96 ^a	4.78	0.51	0.74
	Yes ^g	22.40	16.15	78.87	47.32	24.14 ^b	5.67	0.65	1.10

^{a, b, c} Within a column, means with different superscripts differ ($P < 0.001$; F-test protected LSD: 3.20 for propionate concentration).

^d Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^e Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^f Cultures contained ruminal fluid collected from animals not fed quebracho tannin supplement.

^g Cultures contained ruminal fluid collected from animals fed quebracho tannin supplement.

^h BCVFA means branched-chain volatile fatty acids (isobutyrate and isovalerate).

Table A.6 Effects of culture substrate, antimicrobial, and prior dietary tannin exposure on total gas pressure and ammonia, total VFA, and acetate concentrations following 48-h *in vitro* incubation.

Antimicrobial agent	Culture substrate	Prior tannin exposure	Total gas pressure (psi)	NH ₃ (mM)	Total VFA (mM)	Acetate (mM)
No antimicrobial	Tannin-free ^c	No ^e	33.01	19.92	97.97	63.96
		Yes ^f	28.16	20.95	94.23	56.97
	High-tannin ^d	No ^e	12.78	18.78	58.41	38.72
		Yes ^f	15.82	16.17	68.79	40.70
Penicillin + streptomycin ^a	Tannin-free ^c	No ^e	17.61	20.93	59.49	48.73
		Yes ^f	15.55	21.21	54.98	42.55
	High-tannin ^d	No ^e	9.98	19.50	42.17	33.40
		Yes ^f	8.72	19.04	41.15	30.67
Cycloheximide ^b	Tannin-free ^c	No ^e	29.11	17.40	93.59	60.78
		Yes ^f	28.21	17.52	89.54	54.23
	High-tannin ^d	No ^e	12.90	17.33	57.14	37.98
		Yes ^f	16.59	14.78	68.19	40.41

^a Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^b Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^c Culture substrate consisted only of ground bromegrass hay, no quebracho tannin was present.

^d Culture substrate was 10.2% condensed tannins in the form of quebracho tannin and 89.8% ground bromegrass hay.

^e Cultures contained ruminal fluid collected from animals not fed quebracho tannin supplement.

^f Cultures contained ruminal fluid collected from animals fed quebracho tannin supplement.

Table A.7 Effects of culture substrate, antimicrobial, and prior dietary tannin exposure on propionate, butyrate, valerate, and total branched-chain VFA (isobutyrate + isovalerate) concentrations following a 48-h *in vitro* incubation.

Antimicrobial agent	Culture substrate	Prior tannin exposure	Propionate (mM)	Butyrate (mM)	Valerate (mM)	BCVFA ^k (mM)
No antimicrobial	Tannin-free ^g	No ⁱ	25.26 ^{bc}	6.75	0.70	1.31
		Yes ^j	27.15 ^c	7.42	0.89	1.79
	High-tannin ^h	No ⁱ	14.82 ^a	3.89	0.42	0.56
		Yes ^j	21.66 ^b	4.98	0.46	0.95
Penicillin + streptomycin ^e	Tannin-free ^g	No ⁱ	6.19 ^d	3.47	0.20	0.90
		Yes ^j	7.25 ^d	3.73	0.25	1.20
	High-tannin ^h	No ⁱ	5.36 ^d	2.66	0.21	0.54
		Yes ^j	6.18 ^d	3.12	0.26	0.92
Cycloheximide ^f	Tannin-free ^g	No ⁱ	25.06 ^{bc}	6.06	0.66	1.03
		Yes ^j	26.17 ^{bc}	6.87	0.83	1.45
	High-tannin ^h	No ⁱ	14.85 ^a	3.50	0.36	0.44
		Yes ^j	22.10 ^b	4.47	0.47	0.76

^{a, b, c, d} Within a column, means with different superscripts differ ($P < 0.001$; F-test protected LSD: 3.77 for propionate concentration).

^e Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^f Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^g Culture substrate consisted only of ground bromegrass hay, no quebracho tannin was present.

^h Culture substrate was 10.2% condensed tannins in the form of quebracho tannin and 89.8% ground bromegrass hay.

ⁱ Cultures contained ruminal fluid collected from animals not fed quebracho tannin supplement.

^j Cultures contained ruminal fluid collected from animals fed quebracho tannin supplement.

^k BCVFA means branched-chain volatile fatty acids (isobutyrate and isovalerate).

Appendix B - *In Vitro* Rate of Digestion Tables

Table B.1 Effects of donor-species inoculum and antimicrobial during a 48-h *in vitro* incubation.

Animal species	Antimicrobial agent	IVDMD ^k (%)	NH ₃ (mM)	Total VFA (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	Valerate (mM)	BCVFA ^l (mM)
Bos taurus ^h	No antimicrobial	11.81 ^a	16.55	53.21	34.87	12.28	4.31 ^a	0.53 ^a	1.22
	Penicillin + streptomycin ⁱ	13.34 ^{ad}	17.14	40.87	29.30	6.22	3.70 ^b	0.38 ^b	1.26
	Cycloheximide ^j	12.40 ^a	17.21	51.14	33.60	12.05	3.92 ^c	0.48 ^c	1.09
Ovis aries ^h	No antimicrobial	18.68 ^b	17.13	48.78	32.03	12.59	3.26 ^d	0.30 ^{dg}	0.61
	Penicillin + streptomycin ⁱ	15.48 ^{cd}	18.07	37.99	27.87	6.81	2.47 ^e	0.20 ^e	0.64
	Cycloheximide ^j	16.68 ^{bcd}	16.85	48.09	31.48	12.68	3.10 ^{df}	0.28 ^{fg}	0.54
Capra hircus ^h	No antimicrobial	14.41 ^{acd}	18.78	46.55	31.08	11.47	3.04 ^f	0.31 ^d	0.66
	Penicillin + streptomycin ⁱ	12.13 ^a	19.85	35.27	26.34	5.80	2.25 ^e	0.19 ^e	0.70
	Cycloheximide ^j	15.86 ^d	18.28	45.16	30.18	11.31	2.81 ^g	0.29 ^{fg}	0.57

a, b, c, d, e, f, g Within a column, means with different superscripts differ ($P < 0.001$; F-test protected LSD ($P = 0.001$): 3.0863 for IVDMD, 0.2221 for butyrate concentration, and 0.0260 for valerate concentration).

^h n = 3/species.

ⁱ Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^j Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^k IVDMD means *in vitro* dry matter disappearance.

^l BCVFA means branched-chain volatile fatty acids (isobutyrate and isovalerate).

Table B.2 Effects of donor-species inoculum and culture substrate during a 48-h *in vitro* incubation.

Animal species	Culture substrate	IVDMD ⁱ (%)	NH ₃ (mM)	Total VFA (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	Valerate (mM)	BCVFA ^j (mM)
<i>Bos taurus</i> ^f	Tannin-free ^g	14.39	18.05 ^{bd}	52.39	35.11	11.16 ^{bd}	4.28	0.52	1.32 ^b
	High-tannin ^h	10.64	15.89 ^a	44.42	30.08	9.21 ^a	3.67	0.40	1.06 ^a
<i>Ovis aries</i> ^f	Tannin-free ^g	19.57	17.52 ^{bc}	50.26	33.71	12.26 ^b	3.30	0.31	0.68 ^d
	High-tannin ^h	14.33	17.18 ^c	39.64	27.21	9.13 ^a	2.58	0.21	0.51 ^c
<i>Capra hircus</i> ^f	Tannin-free ^g	15.97	19.11 ^e	47.11	32.26	10.80 ^d	3.01	0.30	0.74 ^e
	High-tannin ^h	12.30	18.82 ^{de}	37.54	26.13	8.25 ^c	2.39	0.22	0.55 ^c

^{a, b, c, d, e} Within a column, means with different superscripts differ ($P < 0.001$; F-test protected LSD ($P = 0.001$): 0.9745 for NH₃ concentration, 0.6023 for propionate concentration, and 0.0419 for BCVFAs).

^f n = 3/species.

^g Culture substrate consisted only of ground bromegrass hay, no quebracho tannin was present.

^h Culture substrate was 10.2% condensed tannins in the form of quebracho tannin and 89.8% ground bromegrass hay.

ⁱ IVDMD means *in vitro* dry matter disappearance.

^j BCVFA means branched-chain volatile fatty acids (isobutyrate and isovalerate).

Table B.3 Effects of culture substrate and prior dietary tannin exposure during a 48-h *in vitro* incubation.

Culture substrate	Prior tannin exposure	IVDMD ^g (%)	NH ₃ (mM)	Total VFA (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	Valerate (mM)	BCVFA ^h (mM)
Tannin-free ^c	No ^e	20.41	19.24	51.65 ^b	36.26 ^b	10.92	3.32	0.36	0.79
	Yes ^f	12.88	17.21	48.18 ^b	31.13 ^{ab}	11.90	3.73	0.39	1.04
High-tannin ^d	No ^e	14.98	18.03	41.01 ^a	29.36 ^a	8.20	2.61	0.25	0.59
	Yes ^f	9.86	16.56	40.06 ^a	26.26 ^a	9.53	3.15	0.30	0.82

^{a, b} Within a column, means with different superscripts differ ($P < 0.001$; F-test protected LSD ($P = 0.001$): 5.7280 for total VFAs, and 5.6418 for acetate concentration).

^c Culture substrate consisted only of ground bromegrass hay, no quebracho tannin was present.

^d Culture substrate was 10.2% condensed tannins in the form of quebracho tannin and 89.8% ground bromegrass hay.

^e Cultures contained ruminal fluid collected from animals not fed quebracho tannin supplement.

^f Cultures contained ruminal fluid collected from animals fed quebracho tannin supplement.

^g IVDMD means *in vitro* dry matter disappearance.

^h BCVFA means branched-chain volatile fatty acids (isobutyrate and isovalerate).

Table B.4 Effects of fermentation time and donor-species inoculum during a 48-h *in vitro* incubation.

Animal species	Incubation time	IVDMD ^d (%)	NH ₃ (mM)	Total VFA (mM)	Acetate (mM)	Propionate (mM)	Butyrate (mM)	Valerate (mM)	BCVFA ^e (mM)
<i>Bos taurus</i> ^c	24 h	14.03	18.22 ^a	51.79	34.70	11.20	4.23	0.49	1.18 ^a
	48 h	19.61	17.93 ^a	55.52	36.43	12.79	4.43	0.52	1.34 ^a
<i>Ovis aries</i> ^c	24 h	17.41	17.46 ^a	48.06	32.26	11.80	3.18	0.28	0.54 ^b
	48 h	23.14	18.34 ^a	50.60	33.04	13.32	3.21	0.31	0.72 ^b
<i>Capra hircus</i> ^c	24 h	15.65	18.96 ^a	44.67	30.60	10.37	2.85	0.27	0.59 ^{ab}
	48 h	19.64	15.66 ^b	50.56	34.05	12.07	3.29	0.34	0.80 ^{ab}

^{a, b} Within a column, means with different superscripts differ ($P < 0.001$; F-test protected LSD ($P = 0.001$): 2.8362 for NH₃ concentration and 0.6049 for BCVFAs).

^c $n = 3$ /species.

^d IVDMD means *in vitro* dry matter disappearance.

^e BCVFA means branched-chain volatile fatty acids (isobutyrate and isovalerate).

Table B.5 Effects of fermentation time, culture substrate, and antimicrobial on IVDMD and ammonia, total VFA, and acetate concentrations during a 48-h *in vitro* incubation.

Antimicrobial agent	Culture substrate	Incubation time	IVDMD ^k (%)	NH ₃ (mM)	Total VFA (mM)	Acetate (mM)
No antimicrobial	Tannin-free ⁱ	24 h	19.45	17.22	59.55 ^b	38.45 ^b
		48 h	28.19	17.35	67.13 ^c	42.28 ^c
	High-tannin ^j	24 h	13.43	17.74	46.37 ^a	30.48 ^{ad}
		48 h	17.44	17.21	49.38 ^a	31.34 ^a
Penicillin + streptomycin ^g	Tannin-free ⁱ	24 h	14.73	20.67	41.62 ^e	30.55 ^{ad}
		48 h	20.32	20.06	46.32 ^a	34.41 ^f
	High-tannin ^j	24 h	12.92	17.94	37.88 ^d	28.11 ^{de}
		48 h	15.09	15.66	36.25 ^d	26.88 ^c
Cycloheximide ^h	Tannin-free ⁱ	24 h	20.16	16.65	59.07 ^b	38.24 ^b
		48 h	26.91	17.33	69.50 ^c	43.79 ^c
	High-tannin ^j	24 h	13.49	19.02	44.55 ^a	29.28 ^{ade}
		48 h	16.83	16.26	44.76 ^a	29.34 ^{ade}

^{a, b, c, d, e, f} Within a column, means with different superscripts differ ($P < 0.001$; F-test protected LSD ($P = 0.001$): 4.3718 for total VFAs, and 3.0261 for acetate concentration).

^g Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^h Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

ⁱ Culture substrate consisted only of ground bromegrass hay, no quebracho tannin was present.

^j Culture substrate was 10.2% condensed tannins in the form of quebracho tannin and 89.8% ground bromegrass hay.

^k IVDMD means *in vitro* dry matter disappearance.

Table B.6 Effects of fermentation time, culture substrate, and antimicrobial on propionate, butyrate, valerate, and branched-chain VFA (isobutyrate +isovalerate) concentrations during a 48-h *in vitro* incubation.

Antimicrobial agent	Culture substrate	Incubation time	Propionate (mM)	Butyrate (mM)	Valerate (mM)	BCVFA ^q (mM)
No antimicrobial	Tannin-free ^o	24 h	15.50 ^c	4.23 ^c	0.49 ^b	0.89 ^c
		48 h	18.13 ^d	4.77 ^d	0.61 ^c	1.34 ^d
	High-tannin ^p	24 h	11.52 ^a	3.38 ^a	0.31 ^{ae}	0.67 ^a
		48 h	13.45 ^b	3.44 ^a	0.36 ^a	0.80 ^b
Penicillin + streptomycin ^m	Tannin-free ^o	24 h	6.76 ^{ef}	3.09 ^{ae}	0.27 ^{de}	0.95 ^g
		48 h	7.27 ^f	3.33 ^a	0.27 ^{de}	1.04 ^h
	High-tannin ^p	24 h	5.97 ^{ef}	2.77 ^e	0.26 ^{de}	0.77 ^e
		48 h	5.56 ^c	2.79 ^c	0.24 ^d	0.78 ^f
Cycloheximide ⁿ	Tannin-free ^o	24 h	15.62 ^c	4.02 ^c	0.45 ^b	0.75 ^k
		48 h	19.30 ^d	4.69 ^d	0.58 ^c	1.15 ^l
	High-tannin ^p	24 h	11.36 ^a	3.03 ^{ae}	0.29 ^e	0.59 ⁱ
		48 h	12.66 ^a	2.83 ^e	0.29 ^e	0.61 ^j

a, b, c, d, e, f, g, h, i, j, k, l Within a column, means with different superscripts differ ($P < 0.001$; F-test protected LSD ($P = 0.001$): 1.4701 for propionate concentration, 0.4417 for butyrate concentration, 0.0518 for valerate concentration, and 0.123 for BCVFA concentration).

^m Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

ⁿ Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^o Culture substrate consisted only of ground bromegrass hay, no quebracho tannin was present.

^p Culture substrate was 10.2% condensed tannins in the form of quebracho tannin and 89.8% ground bromegrass hay.

^q BCVFA means branched-chain volatile fatty acids (isobutyrate and isovalerate).

Table B.7 Effects of donor-species inoculum, culture substrate, antimicrobial, and prior dietary tannin exposure on IVDMD and ammonia, total VFA, and acetate concentrations during a 48-h *in vitro* incubation.

Animal species	Antimicrobial agent	Culture substrate	Prior tannin exposure	IVDMD ^m (%)	NH ₃ (mM)	Total VFA (mM)	Acetate (mM)
<i>Bos taurus</i> ^f	No antimicrobial	Tannin-free ⁱ	No ^k	14.6	19.3 ^{acde}	54.9	36.8
			Yes ^l	12.5	15.3 ^{bcd}	59.7	38.1
		High-tannin ^j	No ^k	9.8	14.9 ^{bcd}	45.5	30.5
			Yes ^l	10.3	16.6 ^{abcd}	52.7	34.1
	Penicillin + streptomycin ^g	Tannin-free ⁱ	No ^k	16.3	20.0 ^{ae}	38.9	29.2
			Yes ^l	12.7	18.2 ^{abde}	47.3	32.4
		High-tannin ^j	No ^k	13.7	13.5 ^{cd}	34.6	26.0
			Yes ^l	10.6	16.8 ^{abcd}	42.7	29.7
	Cycloheximide ^h	Tannin-free ⁱ	No ^k	16.9	18.1 ^{abd}	54.0	36.0
			Yes ^l	13.5	17.3 ^{abcd}	59.5	38.2
		High-tannin ^j	No ^k	10.7	19.6 ^{ae}	42.6	28.6
			Yes ^l	8.6	13.9 ^{cd}	48.5	31.6
<i>Ovis aries</i> ^f	No antimicrobial	Tannin-free ⁱ	No ^k	27.6	18.1 ^{abd}	59.4	40.1
			Yes ^l	16.0	15.9 ^{bcd}	51.0	31.5
		High-tannin ^j	No ^k	19.0	18.9 ^{abde}	45.4	31.6
			Yes ^l	12.2	15.6 ^{bcd}	39.3	24.9
	Penicillin + streptomycin ^g	Tannin-free ⁱ	No ^k	21.8	20.6 ^{ae}	44.7	33.5
			Yes ^l	12.4	18.4 ^{abd}	36.0	25.5
		High-tannin ^j	No ^k	19.1	16.6 ^{abcd}	38.1	29.3

			Yes ^l	8.6	16.7 ^{abcd}	33.2	23.2
	Cycloheximide ^h	Tannin-free ⁱ	No ^k	23.4	16.3 ^{bcd}	59.2	40.1
Yes ^l			16.2	15.8 ^{bcd}	51.2	31.6	
High-tannin ^j		No ^k	15.8	19.7 ^{abe}	43.3	30.0	
		Yes ^l	11.3	15.6 ^d	38.6	24.3	
<i>Capra hircus</i> ^f	No antimicrobial	Tannin-free ⁱ	No ^k	21.9	19.7 ^{ae}	57.1	39.7
			Yes ^l	11.5	18.4 ^{abde}	48.3	30.0
		High-tannin ^j	No ^k	15.0	20.0 ^{ae}	41.9	30.1
			Yes ^l	9.2	17.1 ^{abcd}	39.0	24.5
	Penicillin + streptomycin ^g	Tannin-free ⁱ	No ^k	19.4	22.2 ^e	41.0	32.1
			Yes ^l	6.5	19.5 ^{ae}	34.4	24.3
		High-tannin ^j	No ^k	16.0	18.0 ^{abcd}	35.9	28.1
			Yes ^l	6.7	19.8 ^{ae}	29.7	21.0
	Cycloheximide ^h	Tannin-free ⁱ	No ^k	21.8	18.9 ^{abde}	55.8	38.9
			Yes ^l	14.7	16.1 ^{abcd}	46.0	28.6
		High-tannin ^j	No ^k	15.5	21.1 ^{ae}	42.0	30.0
			Yes ^l	11.3	17.1 ^{bcd}	36.8	23.2

^{a, b, c, d, e} Within a column, means with different superscripts differ ($P < 0.0001$; F-test protected LSD ($P = 0.001$): 4.0107 for NH_3 concentration).

^f $n = 3$ /species.

^g Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^h Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

ⁱ Culture substrate consisted only of ground bromegrass hay, no quebracho tannin was present.

^j Culture substrate was 10.2% condensed tannins in the form of quebracho tannin and 89.8% ground bromegrass hay.

^k Cultures contained ruminal fluid collected from animals not fed quebracho tannin supplement.

^l Cultures contained ruminal fluid collected from animals fed quebracho tannin supplement.

^m IVDMD means *in vitro* dry matter disappearance.

Table B.8 Effects of donor-species inoculum, culture substrate, antimicrobial, and prior dietary tannin exposure on propionate, butyrate, valerate, and branched-chain VFA (isobutyrate + isovalerate) concentrations during a 48-h *in vitro* incubation.

Animal species	Antimicrobial agent	Culture substrate	Prior tannin exposure	Propionate (mM)	Butyrate (mM)	Valerate (mM)	BCVFA ^h (mM)
<i>Bos taurus</i> ^a	No antimicrobial	Tannin-free ^d	No ^f	12.7	3.9	0.51	1.06
			Yes ^g	13.9	5.3	0.69	1.66
		High-tannin ^e	No ^f	10.7	3.1	0.34	0.81
			Yes ^g	11.8	4.9	0.55	1.36
	Penicillin + streptomycin ^b	Tannin-free ^d	No ^f	5.4	2.9	0.29	1.08
			Yes ^g	7.8	4.9	0.51	1.72
		High-tannin ^e	No ^f	4.9	2.5	0.28	0.86
			Yes ^g	6.8	4.5	0.45	1.40
	Cycloheximide ^c	Tannin-free ^d	No ^f	12.9	3.7	0.46	0.92
			Yes ^g	14.3	5.0	0.64	1.48
		High-tannin ^e	No ^f	10.0	2.8	0.31	0.72
			Yes ^g	11.0	4.2	0.49	1.24
<i>Ovis aries</i> ^a	No antimicrobial	Tannin-free ^d	No ^f	14.5	3.7	0.42	0.69
			Yes ^g	14.8	3.7	0.32	0.72
		High-tannin ^e	No ^f	10.2	2.8	0.26	0.52
			Yes ^g	10.9	2.8	0.19	0.51
	Penicillin + streptomycin ^b	Tannin-free ^d	No ^f	7.4	2.7	0.26	0.77
			Yes ^g	7.3	2.5	0.17	0.64

	Cycloheximide ^c	High-tannin ^e	No ^f	5.8	2.2	0.21	0.57	
			Yes ^g	6.8	2.4	0.17	0.56	
		Tannin-free ^d	No ^f	14.6	3.5	0.39	0.61	
			Yes ^g	15.0	3.7	0.32	0.66	
	High-tannin ^e	No ^f	10.0	2.6	0.25	0.45		
		Yes ^g	11.1	2.6	0.19	0.45		
	<i>Capra hircus</i> ^a	No antimicrobial	Tannin-free ^d	No ^f	12.7	3.6	0.39	0.70
				Yes ^g	13.9	3.3	0.37	0.83
High-tannin ^e			No ^f	8.4	2.7	0.23	0.47	
			Yes ^g	11.0	2.6	0.27	0.64	
Penicillin + streptomycin ^b		Tannin-free ^d	No ^f	5.7	2.4	0.18	0.69	
			Yes ^g	6.8	2.3	0.20	0.88	
		High-tannin ^e	No ^f	4.9	2.2	0.17	0.51	
			Yes ^g	5.8	2.1	0.20	0.71	
Cycloheximide ^c		Tannin-free ^d	No ^f	12.5	3.5	0.36	0.59	
			Yes ^g	13.6	3.0	0.33	0.73	
		High-tannin ^e	No ^f	8.8	2.6	0.22	0.40	
			Yes ^g	10.6	2.3	0.24	0.56	

^a n = 3/species.

^b Cultures contained 62.5 mg penicillin (1,600 U/mg; PEN-K; Sigma-Aldrich Chemical Co., Milwaukee, WI) and 10.0 mg streptomycin sulfate (720 IU/mg; S-6501, Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^c Cultures contained 25.0 mg cycloheximide (C7698; Sigma-Aldrich Chemical Co., Milwaukee, WI) in 5 mL of deionized water per culture.

^d Culture substrate consisted only of ground bromegrass hay, no quebracho tannin was present.

^e Culture substrate was 10.2% condensed tannins in the form of quebracho tannin and 89.8% ground bromegrass hay.

^f Cultures contained ruminal fluid collected from animals not fed quebracho tannin supplement.

^g Cultures contained ruminal fluid collected from animals fed quebracho tannin supplement.

^h BCVFA means branched-chain volatile fatty acids (isobutyrate and isovalerate).