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*Corresponding author

Terry E Engle, Colorado State University, Department of Animal Sciences, Fort Collins, CO, 80523, USA

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Research Article

The Influence of Tannins, Saponins, and Direct Fed Microbials on *In Vitro* Rumen Fermentation Characteristics and Microbiota

Arquimides Reyes[1], Huey Yi Loh[2], Meghan P Thorndyke[2], Octavio Guimaraes[2], Richard Goodall[3], Robert J Delmore[2], Keith E Belk[2] and Terry E Engle[2*]

[1]*University of Wisconsin-River Falls, Department of Animal and Food Science, River Falls, WI 54022, USA*

[2]*Colorado State University, Department of Animal Sciences, Fort Collins, CO, 80523, USA*

[3]*MicroBios, Houston, TX, 77002, USA*

Abstract

In-vitro experiments were conducted to determine the impact of tannins, saponins, and Direct-Fed-Microbials (DFM) on rumen fermentation characteristics. Rumen fluid was collected 2h post feeding from three cannulated crossbred (Angus x Hereford) feedlot steers (450±12.2 kg), adjusted to finishing diet. Rumen fluid from all steers were filtered, combined, and mixed with buffer. Vaccine bottles containing ground finishing diet and appropriate treatments were incubated with rumen fluid mixture (39 ˚C). Treatments consisted of 1) Control; 2) Commercial Tannin (TCH); 3) Hydrolysable Tannin (HT); 4) saponin; 5) TCH+DFM; 6) HT+DFM; and 7) Saponin+DFM. At 12 h post incubation, HT+DFM and Saponin+DFM had lesser (P<0.05) molar proportions of acetic acid when compared to all other treatments. Saponin+DFM had greater (P<0.05) molar proportions of propionic acid and a lesser (P<0.05) acetate: propionate ratio when compared to treatments not containing DFM. Dry matter digestibility and pH were greater (P<0.05) for saponin, TCH+DFM, HT+DFM and saponin+DFM, whereas pH was greater in treatments containing DFM compared to the controls. Percent CO_2 was lesser (P<0.05) in saponin and THC+DFM *in vitro* vessels compared to controls. At 18 h post fermentation total VFA concentrations, molar proportions of butyric and valeric acid, and gas cap N_2 percentage were greater (P<0.05) in *in vitro* vessels containing HT+DFM compared to controls. The percentage of CO_2 was lesser in *in vitro* vessels containing HT compared to control and *in vitro* vessels containing saponin, HT+DFM and Saponin+DFM. At 12 h, alpha and beta diversity of the microbial community between treatments differed (P<0.05). Across all samplesat 18 h of fermentation the genera Sharpea and Dialister were present, however Saponin+DFM and TCH+DFM treatments demonstrated the greatest relative abundance. While Sharpea contribute to rumen biohydrogenation, the presence of genus Dialister indicates association with energy harvesting bacteria and suggests that the inclusion of several of these feed additives allowed shifts of certain bacteria to occur.

Introduction

The digestive track of ruminants is designed for digestion of a variety of feed sources, in part, via microbial fermentation which yields Volatile Fatty Acids (VFA) [1]. Certain byproducts of fermentation are absorbed through the ruminal epithelium and provide the animal with substrates for energy metabolism. As dietary fermentable starch increases, there is a shift in the rumen microbial community from cellulolytic digesting bacteria to more amylolytic digesting bacteria [2]. A shift from cellulolytic to amylolytic bacteria in the rumen increases the production of total VFA as well as increases the molar proportions of propionate while decreasing molar proportions of acetate. This dynamic shift in VFA by increasing the readily fermentable starch in the diet, increases growth efficiency of the animal but also reduces ruminal pH. These changes in pH can cause alterations in digestion leading to acute reductions in rumen pH below 5.1- resulting in acidosis. Ruminal acidosis compromises the integrity of the ruminal epithelium and permits bacterial translocation to the blood stream, which ultimately may cause liver abscesses [3]. Liver abscesses result in substantial economic loss to the industry [4]. Producers incur losses due to decreased feed efficiency, reduction of feed intake, lower weight gain, and ultimately decreased carcass dressing percentage. Furthermore, the packing industry can incur economic losses due to condemnation of the liver [5]. Brown and Lawrence [6] reported that a 3.5 % incidence rate of liver abscesses would cost the industry over $7 million annually. To prevent liver abscess formation, the beef cattle industry utilizes feed grade antibiotics. The most commonly used feed grade antibiotic is tylosin phosphate. Tylosin phosphate is classified as a macrolide antibiotic, which are important for clinical treatment of human infections [7]. Therefore, there is a concern that feeding tylosin phosphate to beef cattle could lead to increased levels of macrolide antibiotic resistance in humans, ultimately leading to treatment failures [8].

The public concern surrounding antibiotic resistance has led to an on-going investigation of alternative technologies for decreasing the incidence of liver abscesses without the use of antibiotics. Plant compounds, such as saponins and tannins, are used by plants as self-defense mechanisms to prevent predation and disease [9] and may be efficacious in reducing ruminal acidosis and preventing liver abscesses in beef cattle. These compounds have been reported to improve rumen health [10-13] and fermentation characteristics [9,14-18] by optimizing starch digestibility and maintaining rumen pH, ultimately improving ruminal wall integrity and reducing the overall incidence of liver abscesses. The use of Direct-Fed Microbials (DFM) has also been reported to enhance animal efficiency by altering ruminal bacterial communities [19]. Research has demonstrated that the inclusion of DFM in feedlot diets can reduce shedding of enteric bacteria by feedlot cattle [20]. Even though DFM and plant compounds have been shown to positively benefit the animal, the impacts of DFM and

plant compounds on ruminal fermentation characteristic and microbiota populations are difficult to elucidate. Due to production changes and consumer sentiment, it is critical to explore new alternatives in order to increase animal efficiency and decrease liver abscess rate. Therefore, the objective of this study was to evaluate the effects of saponins, tannins and DFM on rumen fermentation characteristics and ruminal microbiota composition *in vitro*.

Materials and Methods

Animals were utilized in accordance with Colorado State University's (CSU) Institutional Animal Care and Use Committee (IACUC) approval (Protocol 16-6550A). Steers were housed at CSU's Agricultural Research, Development and Education Center (AR-DEC).

Rumen content collection

Three crossbred feedlot steers (Angus x Hereford; 450 kg±12.2 kg; ~3.0 years of age) fitted with ruminal fistulas were utilized in this study. Prior to sample collection, cattle were adjusted to a high energy finishing grain-based diet (1.43 NEg, Mcal/kg DM) for 3 weeks (21 d; Table 1). At the completion of the 21-d diet adjustment period, rumen fluid was collected at a single time point, approximately 2 h post feeding, as described by Ward and Spears [21]. Briefly, rumen fluid (~4 L) from all three steers was filtered twice through four layers of cheesecloth and combined into one pre-warmed (39 °C) thermos. A modified McDougall's buffer solution (39.20 g $NaHCO_3$, 14.80 g Na_2HPO_4, 2.28 g KCl, 1.88 g NaCl, 0.48 g $MgSO_4$*$7H_2O$ per 2 L H_2O) was mixed with rumen fluid at a 1:1 ratio, simulating saliva production during rumination [22]. Rumen fluid pH was recorded using a HI98190 handheld pH meter (EcoTestr pH 2+; Oaktron 153 Instruments, Vernon Hills, IL, USA) before and after being mixed with McDougall's buffer solution.

Table 1: Ingredients and chemical composition of the basal diet in Dry Matter (DM) basis.

Item	Percentage
Ingredient Composition (% DM)	
Steam Flaked Corn	61
Corn Silage	14
Alfalfa Hay	10
Dry Distillers Grain (DDG)	10
Fat (Tallow)	5
Chemical Composition	
Dry Matter, %	69.8
Crude Protein, %	12.9
ADF, %[1]	9.5
NDF, %[2]	16.3
NEg, Mcal/kg[3]	1.4

Macro- and microminerals included in diet: calcium=0.71%, phosphorus= 0.33%, salt 0.51%, potassium 0.62%, sulfur 0.16%, magnesium 0.18%, zinc 19.50 ppm, iron 169.42 ppm, copper 6.04 ppm, manganese 11.80 ppm, cobalt 0.12 ppm, iodine 0.50 ppm, selenium 0.13 ppm, sodium 0.24%, and chlorine 0.44%.

1. ADF=Acid detergent fiber.
2. NDF=Neutral detergent fiber.
3. NEg=Net energy for growth.

In vitro chambers

Approximately 2 kg (wet weight) of the high concentrate diet fed to the steers was collected upon discharge from the feed truck and dried in a forced air-drying oven at 60 °C for 72 h and ground through a 2.0 mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ). The ground ration was weighed and dispensed (1.000±0.005 g) into pre-labeled 100 mL vaccine bottles containing the appropriate dose of each treatment (n=20/treatment; N=140). Treatments consisted of: 1) Control (finishing diet+rumen fluid/McDougall's buffer mixture); 2) commercial tannin (TCH; Condensed + Hydrolysable; 15 g/steer equivalent); 3) hydrolysable tannin (HT; 15 g/steer equivalent); 4) saponin (*Yucca schidigera*; 2g/steer equivalent); 5) commercial tannin+direct-fed-microbial (TCH+DFM;15 g/steer equivalent of TCH and $2x10^8$+$2.50x10^7$ Colony-Forming Units (CFU)/g/steer equivalent of DFM) of *Propionibacterium acidipropionici* and *Lactobacillus animalis*); 6) Hydrolysable Tannin+DFM (HT+DFM); and 7) Saponin+DFM. Two sets of 10 vaccine bottles were prepared for each treatment per time point. One set of vaccine bottles was used to evaluate rumen fermentation characteristics at two time points (12 h and 18 h), while the second set was used for microbiome analysis at the same time points.

The McDougall's buffer/rumen fluid mixture was dispensed into the vaccine bottles (50 mL) containing the pre-weighed feed and treatment. The bottles were purged with CO_2 for 10 seconds, capped and sealed immediately after the McDougall's buffer/rumen mixture was dispensed. The vaccine bottles remained sealed to maintain anaerobic conditions and were incubated at 39 °C in a circulating water bath for the remainder of the experiment. Vaccine bottles were removed for sampling purposes as required by the experimental design.

Sample collection

To simulate rumen motility, vaccine bottles were gently swirled every 4 h. Samples were removed at each predetermined time point, gas pressure was recorded, and a 10 mL gas sample was collected and immediately analyzed for nitrogen (N_2), methane (CH_4) and carbon dioxide (CO_2) concentrations (mL) via gas chromatography. At each collection time, the pH of the contents of each vaccine bottle destined for analysis of rumen fermentation characteristics was measured. Then, contents of each vaccine bottle were transferred to individual pre-weighed 50 mL conical tubes and centrifuged at 1,000xg at 5 °C for 30 min (Beckman Model TJ-6; Beckman Coulter, Indianapolis, IN). A 2 mL aliquot of the supernatant was extracted from the conical tube post centrifugation, acidified with meta-phosphoric acid, and frozen at -80 °C until analyzed for Volatile Fatty Acid (VFA) concentrations via gas chromatography. The remaining supernatant was aspirated, and the indigestible residue was dried in a forced air-drying oven at 60 °C for 120 h to determine *in vitro* Dry Matter Disappearance (DMD). The second set of paired vaccine bottles were immediately removed at each collection time period and dispensed into 50 mL conical tubes for 16S rRNA gene amplicon sequencing.

Volatile fatty acid analysis

After thawing at room temperature, samples designated for VFA analysis were centrifuged at 28,000xg at 5 °C for 15 min and the supernatant was removed and placed into a 1.5 mL gas chromatography vial and analyzed for VFA's. The VFA concentrations were determined via gas chromatography (Agilent 6890N, Santa Clara, CA) fitted with a fused silica capillary column (30 m x 0.25 µm x 0.25 µm) and a flame ionization detector. The following instrument parameters were used: injection mode=spitless; injection volume=1.0 µL; carrier gas=helium; carrier gas flow=1.0 Ml/min; injector temperature=250 °C; oven ramping program=100 °C for 3 min, 185°C for 11 min; detector temperature 250 °C.

In vitro gas production analysis

Gas pressure of each vaccine bottle was determined using a digital pressure gauge fitted with a 20-gauge needle (Dwyer Instruments Inc., Michigan City, IN). Gas composition (N_2, CH_4, and CO_2) was determined by withdrawing 10 mL of gas from the vaccine bottle headspace using a 10 mL syringe. The gas samples were immediately injected into the injection port of a gas chromatograph (Shimadzu GC-14A; Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector set at 100 °C.

Dry matter disappearance

The Dry Matter Disappearance (DMD) was determined at 12 h and 18 h by weighing the 50 mL conical tubes prior to dispensing the vaccine bottle rumen contents into the tube. An aliquot of the supernatant was used for VFA analysis, as described above, while the solid undigested material was allowed to dry in a forced air-drying oven at 60 °C for 120 h. Once the sample was dry, the conical tube was weighed again to determine the remaining feed that was not digested, and DM disappearance was calculated.

Rumen fermentation characteristics statistical analysis

This experiment was designed as a randomized complete block design replicated over two days. Data were blocked by hour of sample collection. Least squares means and pooled Standard Error of the Mean (SEM) were reported for all response variables. Hour after feeding prior to rumen fluid collection was used as a covariate, due to the steers being fed three hours earlier on the second day of rumen collection as compared to the first rumen fluid collection. Data were analyzed using the Mixed Procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). When a significant treatment effect was detected, treatment means were separated using the PDIFF option of the LSMEANS statement of SAS. (SAS Inst. Inc., Cary, NC). Differences were reported at a significance level of α=0.05.

16S amplicon sequencing

At 12 and 18 hours, 10 artificial rumen samples per treatment were removed from the water bath and transferred into a 50 mL conical tube. The samples were placed on ice and transported to a -80 ˚C freezer until DNA extraction. Prior to DNA extraction, samples were thawed. A sterile cotton swab was used to swab a homogeneous mixture of rumen fluid and the swab was used in the QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany). After DNA extraction, qualified DNA libraries from each sample were shipped to Novogene Corporation (Beijing, China) for 16S rRNA library preparation and sequencing. The V4 region of the 16S subunit was amplified with the 515/806R primer set. Paired-end sequencing (2 x 250) was conducted on an Illumina HiSeq 2500 (Illumina, Inc., San Diego, CA).

Bioinformatics and statistics

Amplicon samples were processed through the QIIME2 (2018.8) pipeline [23]. Samples were demultiplexed and assigned Exact Sequence Variance (ESV) using the DADA2 plugin with primers and lower quality ends trimmed off [24]. Multiple sequence alignments of the sequences were completed with MAFFT and filtered to remove highly variable positions [25]. FastTree 2 was used to construct and root a phylogenetic tree [26]. Taxonomic classification was conducted using a pretrained Naive Bayes classifier trained on the Greengenes database trained with the 515/806 primers [27]. Reads assigned to mitochondria and chloroplast were removed from downstream analysis as well as those reads that did not have an assignment to phylum. Alpha diversity was assessed via Faith's Phylogenetic Diversity and beta diversity was measured using weighted UniFrac distances. Alpha diversity was compared using the lm and anova functions in R and compared using the CLD function from the em means package. Beta diversity was evaluated using the PERMANOVA adonis plugin in QIIME2. Differential abundance was conducted at the aggregated phylum level via ANCOM [28]. In all comparisons, α=0.05 and an FDR adjustment was used when appropriate.

Results

Rumen fermentation characteristics

12 h of fermentation: Treatment effects on VFA concentrations for 12 h of fermentation are presented in Table 2. Total VFA concentrations were similar (P=0.11) across treatments. However, molar proportions of acetic acid and the acetic acid:propionic acid ratio were lesser (P<0.05) in *in vitro* tubes containing HT+DFM compared to controls. *In vitro* vessels containing saponin+DFM had greater (P<0.05) molar proportions of propionic acid when compared to controls but similar molar proportions of propionic acid when compared to other treatments containing DFM. Treatments containing DFM had a greater (P<0.05) DMD compared to treatments not containing DFM. *In vitro* vessel pH was greater in HT+DFM and saponin+DFM when compared to control and TCH treatments. Saponin and treatments with DFM produced less (P<0.05) gas when compared to control (Table 3) at 12 h post incubation. At 12 h post incubation, gas cap pressure per unit DM digested was lesser (P<0.05) in saponin and THC+DFM treatments when compared to control, TCH, and HT treatments. A higher percentage of CO_2 was found for HT (P<0.05) than all other treatments except TCH. Furthermore, methane concentration was lesser (P<0.06) for HT and TCH when compared to all other treatments (Table 3).

Table 2: Effect of direct-fed microbials (DFM), saponins, and tannins on 12 *h in vitro* rumen fermentation characteristics.

Item	Treatment								P <
	Control[1]	TCH[2]	HT[3]	Saponin[4]	TCH + DFM[5]	HT + DFM[6]	Saponin +DFM	SEM[7]	Trt[8]
Total VFA, mM[9]	87.62	88.2	87.01	86.37	87.99	89.96	89.36	0.92	0.11
Acetic Acid, mM/100 mM	48.03^{a}	47.91^{a}	49.85^{a}	47.60^{ab}	45.77^{abc}	38.22^{c}	38.87^{bc}	3.08	0.05
Propionic Acid, mM/100 mM	28.82^{bc}	26.59^{c}	28.46^{c}	29.28^{bc}	31.03^{abc}	35.62^{ab}	37.74^{a}	2.42	0.02
Butyric Acid, mM/100 mM	21.1	22.64	21.66	20.71	21.37	23.83	20.88	1.03	0.32
Valeric Acid, mM/100 mM	2.05	2.86	1.94	2.12	1.83	2.33	2.51	0.48	0.75
Acetic: Propionic	1.71^{ab}	1.85^{a}	1.79^{a}	1.72^{ab}	1.74^{a}	1.33^{bc}	1.13^{c}	0.14	0.01
DMD, %[10]	55.91^{bc}	55.32^{bc}	49.48^{c}	62.65^{ab}	69.47^{a}	66.70^{a}	66.98^{a}	2.62	0.01
pH	5.24^{b}	5.11^{c}	5.34^{ab}	5.31^{ab}	5.35^{ab}	5.40^{a}	5.40^{a}	0.04	0.01

^{a,b,c,d,e}Means with different superscripts within a row differ (P < 0.05).
[1]Control=Feed+rumen fluid/McDougall's buffer mixture (50% rumen fluid: 50% McDougall's buffer).
[2]TCH=Commercial Condensed and Hydrolysable Tannin supplemented at 15 g/steer equivalent.
[3]HT=Hydrolysable Tannin supplemented at 15 g/steer equivalent.
[4]Saponin supplemented at 2g/steer equivalent.
[5]TCH+DFM=Commercial Condensed and Hydrolysable Tannin + Direct Fed Microbial (15 g/steer equivalent of TCH and 2 x 10^8 + 2.50 x 10^7 colony-forming units (CFU/g/steer equivalent of DFM) of *Propionibacterium acidipropionici* and *Lactobacillus animalis*.
[6]HT+DFM=Hydrolysable Tannin+Direct Fed Microbial.
[7]SEM=Pooled standard error of the mean.
[8]Trt=Treatment.
[9]VFA=Volatile fatty acids.
[10]DMD=Dry matter disappearance.

Table 3: Effect of direct-fed microbials (DFM), saponins and tannins on 12 and 18 hour *in vitro* rumen fermentation gas production.

Item	Treatment								P <
	Control[1]	TCH[2]	HT[3]	Saponin[4]	THC+DFM[5]	HT+ DFM[6]	Saponin +DFM	SEM[7]	Trt[8]
12 Hours									
Nitrogen, %	4.62	4.67	4.63	4.3	4.52	4.69	4.63	0.1	0.07
Methane, %	16.29	15.24	14.67	16.71	16.16	15.93	15.91	0.47	0.06
CO_2, %	79.10^{b}	80.08^{ab}	80.70^{a}	78.98^{b}	79.32^{b}	79.38^{b}	79.31^{b}	0.41	0.05
Gas Pressure (kPa)	101.0^{a}	100.5^{ab}	98.9^{abc}	89.1^{e}	92.7^{de}	96.3^{bcd}	95.8^{cd}	15.9	0.01
GP/DMD[9]	28.2^{a}	28.15^{ab}	27.5^{abc}	24.8^{e}	25.9^{de}	26.9^{bcd}	26.7^{cd}	0.43	0.01
18 Hours									
Nitrogen, %	4.54^{bc}	4.37^{c}	4.18^{c}	5.30^{ab}	4.84^{bc}	5.77^{a}	4.83^{bc}	0.31	0.01
Methane, %	20.84	21.88	24.32	19.79	20.04	22.09	18.65	1.26	0.07
CO_2, %	74.62^{ab}	73.75^{abc}	71.49^{c}	74.90^{ab}	75.11^{ab}	72.14^{bc}	76.52^{a}	1.08	0.03
Gas Pressure (kPa)	97.3	93.9	92.6	113.9	104	130.3	124	11.4	0.13
GP/DMD[9]	27.2^{abc}	26.1^{bc}	23.5^{c}	30.8^{ab}	29.5^{abc}	33.1^{a}	32.5^{ab}	2.4	0.07

[a,b,c,d,e]Means with different superscripts within a row differ (P < 0.05).

[1]Control=Feed+rumen fluid/McDougall's buffer mixture (50% rumen fluid:50% McDougall's buffer). [2]TCH= Commercial Condensed and Hydrolysable Tannin supplemented at 15 g/steer equivalent.

[3]HT=Hydrolysable Tannin supplemented at 15 g/steer equivalent.

[4]Saponin supplemented at 2g/steer equivalent.

[5]TCH+DFM=Commercial Condensed and Hydrolysable Tannin+Direct Fed Microbial (15 g/steer equivalent of TCH and 2 x108 + 2.50 x 107 colony-forming units ((CFU)/g/steer equivalent of DFM) of *Propionibacterium acidipropionici* and *Lactobacillus animalis*).

[6]HT+DFM=Hydrolysable Tannin+Direct Fed Microbial.

[7]SEM=Pooled standard error of the mean.

[8]Trt=Treatment.

[9]GP/DMD=Gas pressure per gram of dry matter disappearance

18 h of fermentation: Results for rumen characteristics at 18 h of fermentation characteristic are shown in Tables 3 and 4. There was a significant impact of treatment inclusion on total VFA concentrations (Table 4). Total VFA, butyric acid and valeric acid concentrations were greatest in HT+DFM (P<0.05). However, molar proportions of acetic and propionic acid, the acetic acid:propionic acid ratio, DMD, and pH were not impacted by treatment. At 18 h post incubation the percentage of N_2 measured in the *in vitro* vessel gas cap was lesser (P<0.05) in HT+DFM vessels compared to controls. The percentage of CO_2 was lesser in *in vitro* vessels containing HT compared to control and *in vitro* vessels containing saponin, TCH+DFM and Saponin+DFM. Gas cap pressure was highest for TCH+DFM and lowest for HT treatments (P<0.05), with other treatments being intermediate. The opposite relationship occurred for methane, with TCH+DFM being lower than HT. Methane was also lower for saponin versus HT at 18 h. At 12 h post incubation, gas cap pressure per unit DM digested was lesser (P<0.05) in saponin and THC+DFM treatments when compared to control, TCH, and HT treatments. At 18 h post incubation, gas cap pressure per unit DM digested was lesser (P<0.05) in HT compared to saponin, HT+DFM, and saponin+DFM treatments.

Table 4: Effect of direct-fed microbials (DFM), saponins, and tannins on 18 hours *in vitro* rumen fermentation characteristics.

Item	Treatment								P <
	Control[1]	TCH[2]	HT[3]	Saponin[4]	TCH+DFM[5]	HT+DFM[6]	Saponin + DFM	SEM[7]	Trt[8]
Total VFA, mM[9]	86.46^{b}	87.76^{b}	86.58^{b}	87.57^{b}	87.52^{b}	91.83^{a}	88.92^{ab}	1.11	0.02
Acetic Acid, mM/100 mM	52.36	52.54	52.23	47.71	49.64	42.6	46.02	2.56	0.06
Propionic Acid, mM/100 mM	26.73	26.87	26.04	30.56	27.25	23.52	25.86	1.92	0.31
Butyric Acid, mM/100 mM	18.78^{c}	19.41b^{c}	19.92b^{c}	18.82^{c}	20.02^{bc}	24.79^{a}	21.66^{b}	0.85	0.01
Valeric Acid, mM/100 mM	2.22^{b}	1.59^{b}	1.89^{b}	3.01^{b}	3.18^{b}	9.18^{a}	4.44^{b}	1.25	0.01
Acetic: Propionic	2.34	2.39	2.33	2.01	2.22	2.09	2.15	0.16	0.58
DMD, %[10]	52.98	51.79	56.78	52.45	52.23	54.89	56.45	1.68	0.18
pH	5.56^{c}	5.71^{b}	5.62^{bc}	5.62^{bc}	5.70^{b}	5.90^{a}	5.51^{c}	0.05	0.01

[a,b,c,d,e] Means with different superscripts within a row differ (P < 0.05).

[1]Control = Feed + rumen fluid/McDougall's buffer mixture (50% rumen fluid: 50% McDougall's buffer).

[2]TCH = Condensed and Hydrolysable Tannin supplemented at 15 g/steer equivalent.

[3]HT = Hydrolysable Tannin supplemented at 15 g/steer equivalent.

[4]Saponin supplemented at 2g/steer equivalent.

[5]HT+DFM = HT+DFM (TCH+DFM; 15 g/steer equivalent of TCH and 2 x 10^8 + 2.50 x 10^7 colony forming units (CFU)/g/steer equivalent) of *Propionibacterium acidipropionici* and *Lactobacillus animalis*).

[6]HT+DFM = HT+DFM.
[7]SEM = Pooled standard error of the mean.
[8]Trt = Treatment.
[9]VFA = Volatile fatty acids.
[10]DMD = Dry matter disappearance.

16s sequencing at 12 and 18 hours post fermentation

At 12 hours post fermentation, treatment was a significant source of variation for alpha (P=0.004; Figure 1) and beta diversity (P=0.04; Figure 2) of the microbial community. When phyla were compared, Fusobacteria was greatest (P>0.05) in controls compared to all other treatments (data not shown). The biological ramifications of this numeric difference is unclear as the highest Fusobacteria percentage present was less than 1% of the total phyla present. The predominant phyla found (more than 1% of entire microbiome) were Bacteroides, Firmicutes and Proteobacteria (data not shown). Moreover, the predominant orders represented (more than 1% of entire microbiome) across treatments were Lactobacillales, Clostridiales, Bacteroidales, Enterobacteriales and Erysipelotrichales (Figure 3). At 12 h of fermentation, Saponin+DFM and HT+DFM had a greater mean relative abundance of the order Bacillales (Figure 3). The genus Clostridium was present in a higher abundance in all treatments, except TCH and TCH+DFM (Figure 4). In addition, the genus Proteus and Oscillospira were present in all samples. However, all treatments had a lower mean relative abundance for these two genera when compared to Control (Figure 4).

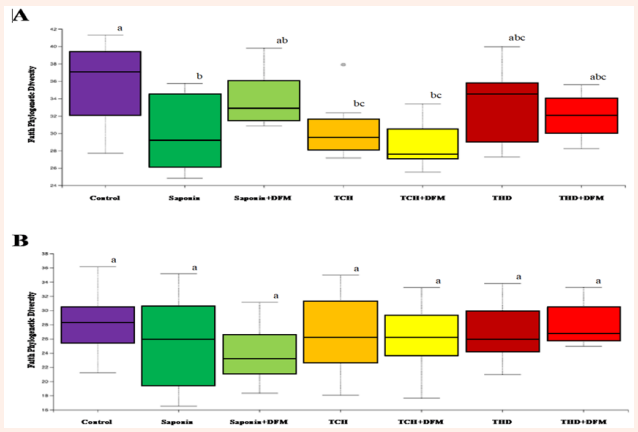

Figure 1: Alpha diversity as measured by Faith's phylogenetic diversity Index. Alpha diversity was significantly (P=0.004) different at 12 h of fermentation (A) and not different (P=0.55) at 18 h of fermentation (B) between all sample types. Statistical differences for alpha diversity among all treatments within times were determined using non-parametric Kruskal-Wallis test.

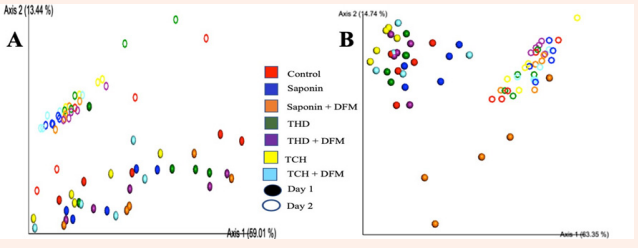

Figure 2: Beta diversity or microbial community composition visualized as principal coordinates analysis ordination bases on 16S rRNA gene sequence by Weighted UniFrac distances color by treatment and shape by day. Treatments at 12 h differ by treatment (P=0.037; A) but did not differ by treatment group at 18 h of fermentation (P=0.13; B).

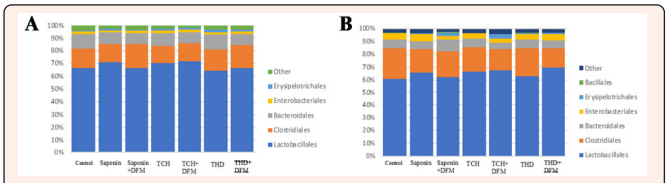

Figure 3: Normalize relative abundance of orders of bacteria (orders present in less than 1% of the entire microbiome were grouped into "other"). 12 h are represented in (A) and 18 h are represented in (B).

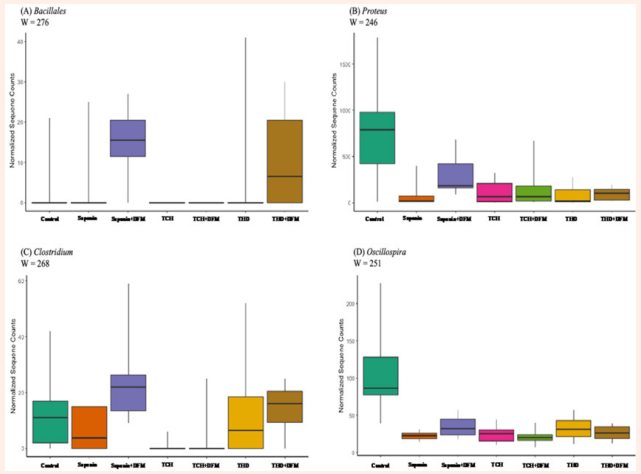

Figure 4: Relative abundance of the differentially abundant microbiota at 12 h of fermentation, (A) Bacillales, (B) Proteus, (C) Clostridium, and (D) Oscillospira, detected by ANCOM between treatments. Results are displayed as mean relative abundance for each treatment, with horizontal black lines delineating the abundance of unique SVs assigned within a given genus.

At 18 h of fermentation, the microbiome between treatments did not differ in either alpha (P=0.55) and beta (P=0.13) diversity as assessed by Faith's phylogenetic diversity (Figure 1) and weighted unifract distances (Figure 2). Moreover, the predominant orders represented (more than 1% of entire microbiome) across treatments were Lactobacillales, Clostridiales, Bacteroidales, Enterobacteriales, Erysipelotrichales, and Bacillales (Figure 3). At 18 h of fermentation, the genus Sharpea was present in higher abundance for TCH+DFM followed by Saponin+DFM (Figure 5). In addition, the genus Dialister was present in Control, Saponin+DFM and TCH+DFM only. Of these treatments, Saponin+DFM had a higher presence of Dialister when compared to all the treatments.

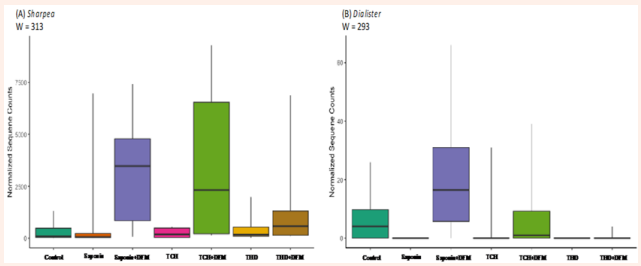

Figure 5: Relative abundance of the differentially abundant microbiota at 18 h of fermentation, (A) Sharpea, and (B) Dialister, detected by ANCOM between treatments. Results are displayed as mean relative abundance for each treatment, with horizontal black lines delineating the abundance of unique SVs assigned within a given genus.

Discussion

Rumen *in vitro* fermentation characteristics

In this experiment the effects of tannins, saponins and DFM were evaluated to determine the impact of these feed additives on rumen fermentation. The inclusion of DFM, in combination with tannins and saponin treatments resulted in greater molar proportions of propionic acid, higher pH and higher rumen DMD. The DFM used in this study consisted of a combination of *Lactobacilus animalis* and *Propionibacterium acidipropionici*. Our results agree with the theory and results of Mandal et al. [28]. These researchers reported that lactic acid producing bacteria can provide enough lactic acid to stimulate growth of lactic acid utilizing bacteria in the rumen. Increasing the population of lactic acid utilizing bacteria results in a decrease in lactic acid and an overall increase in ruminal pH. The addition of *Propionibacterium acidipropionici* in the DFM used in this experiment increased molar proportions of propionic acid at 12 h post fermentation. Greater molar proportions of rumen propionate in response to *Propionibacteria acidipropionici* administration have also been reported by Gifford et al. [29] and Levenson et al. [30] *in vivo*. However, contradicting results were reported by Yang et al. [31] where *Propionibacterium acidipropionici* treatment resulted in less rumen propionate production. Furthermore, in the current experiment, at 12 h of fermentation, the inclusion of saponin alone had no impact on propionic acid production. In previous research, saponin (*Yucca schidigera* extract) has been reported to increase propionic acid production *in vitro* [32,33].

In vitro supplementation of tannins (TCH and HT) tended ($P<0.06$) to reduce methane production at 12 hours in contrast to the previous research where no reduction of methane was shown with the feeding of Chestnut tannins to sheep [9] and Quebracho tannins to cattle [34]. However, other tannin extracts (*Acacia cyanophylla*; vetch-oat hay) have been reported to reduce *in vitro* methane production [35]. At 18 h post *in vitro* fermentation both saponin and saponin+DFM tended ($P<0.07$) to reduce methane production. The reduction of methane has also been observed with supplementation of *Quillaja* and *Yucca schidigera* saponins [35]. Our results suggest that the inclusion of tannins, saponins and DFM show promising traits for altering *in vitro* rumen fermentation characteristics.

Effects of supplementing tannins, saponin, and DFM on rumen microbial community

At both collection time points for all treatments, the majority of phyla present were Bacteroides, Firmicutes and Proteobacteria (data not shown). The presence of these phyla is important as they have been determined to consistently be present in the rumen core microbiome [36]. Even though microbiome comparisons between 12 h and 18 h were not conducted, due to the experimental design not being considered a repeated measures design, the order Enterobacteriales increased as fermentation progressed (Figure 3). The presence of the genus Proteus is ubiquitous to the mammalian digestive tract [37]. The general reduction (Figure 4) of this genus by the test treatments is important, as this bacteria has been found to be transmitted from the fecal matter of birds to cattle feed, which may pose as autoinfection and cross-infection threats [37,38]. Oscillospira is a common inhabitant of the rumen environment [39]. Mackie et al. [40] found that switching from a Lucerne pellet diet to a 70% grain diet drastically reduced the presence of Oscillospira from $1.3x10^5$ to 50 cfu per g of ingesta. Experimental treatments in this experiment resulted in a reduction in Oscillospora (Figure 4). Allison et al. [41] stated that the toxins produced by Clostridium are proteins that rapidly degrade in the rumen. The Commercial Tannin (THC), with or without DFM caused a reduction (Figure 4) in Clostridium, while the HT treatment did not. Petri et al. [36] found that Sharpea increased during acidotic challenges with a grain-based versus forage-based diet, followed by a sharp decline during the recovery period. Similarly, our study exhibited higher abundance of Sharpea for Saponin+DFM and TCH+DFM treatments (Figure 5) in an *in vitro* grain based simulated diet. In addition, the increase of the genus Dialister has been associated with energy harvesting and increased average daily gain [42]. Their higher abundance in the Saponin+DFM treatment (Figure 5) can be associated with higher energy available for growth. This result is supported by the *in vitro* fermentation result for 12 h acetic:propionic and %DMD (Table 2) and 18 h gas pressure and methane concentration results (Table 3).

Conclusion

These data demonstrate a proof of concept for ruminal manipulation resulting in modifying ruminal homeostasis for rumen environments that are subjected to high concentrate diets. As a result, there is a possibility that these test article dietary compounds could raise pH, alter VFA production, and correspondingly reduce bacterial translocation of microorganisms out of the rumen and therefore decrease liver abscess rates. While further data is needed to fully assess the impact of tannins, saponins, and DFM on live animal production, these commercially available compounds and their combinations demonstrate the potential for use in animal production systems.

Author Contributions

Conceptualization, T.E.E., R.J.D., S.R.G, A.R; data collection, A.R, M.P.T, O.G, T.E.E; manuscript preparation, A.R, H.Y.L, M.P.T, O.G, S.R.G, R.J.D, T.E.E. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Prior to the initiation of this experiment, all animal use, handling, and sampling techniques described herein were approved by the Colorado State University Animal Care and Use Committee (Protocol #663; approval date: 27-April-2023).

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