Effect of humic substances on rumen fermentation, nutrient digestibility, methane emissions, and rumen microbiota in beef heifers¹

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ABSTRACT: Ruminants play an important role in food security, but there is a growing concern about the impact of cattle on the environment, particularly regarding greenhouse gas emissions. The objective of this study was to examine the effect of humic substances (HS) on rumen fermentation, nutrient digestibility, methane (CH₄) emissions, and the rumen microbiome of beef heifers fed a barley silage-based diet. The experiment was designed as a replicated 4 × 4 Latin square using 8 ruminally cannulated Angus × Hereford heifers (758 ± 40.7 kg initial BW). Heifers were offered a basal diet consisting of 60% barley silage and 40% concentrate (DM basis) with either 0- (control), 100-, 200- or 300-mg granulated HS/kg BW. Each period was 28 d with 14 d of adaptation. Rumen samples were taken on day 15 at 0, 3, 6, and 12 h postfeeding. Total urine and feces were collected from days 18 to 22. Blood samples were taken on day 22 at 0 and 6 h postfeeding. Between days 26

and 28, heifers were placed in open-circuit respiratory chambers to measure CH₄. Ruminal pH was recorded continuously during the periods of CH₄ measurement using indwelling pH loggers. Intake was similar ($P = 0.47$) across treatments. Concentration of ammonia-N and counts of rumen protozoa responded quadratically ($P = 0.03$), where both increased at H100 and then decreased for the H300 treatments. Apparent total tract digestibility of CP ($P = 0.04$) was linearly increased by HS and total N retention (g/d, % N intake, g/kg BW^{0.75}) was improved ($P = 0.04$) for HS when compared with the control. There was no effect of HS on CH₄ production (g/d; $P = 0.83$); however, HS decreased the relative abundance of Proteobacteria ($P = 0.04$) and increased the relative abundance of Synergistetes ($P = 0.01$) and Euryarchaeota ($P = 0.04$). Results suggest that HS included at up to 300 mg/kg BW may improve N retention and CP digestibility, but there was no impact on CH₄ production.

Key words: beef cattle, fermentation, humic substances, methane, microbiome, rumen

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INTRODUCTION

Research into feed additives that could act as alternatives to antibiotics and hormones is of integral importance as consumers become increasingly apprehensive, concerning the use of these growth-promoting technologies in livestock production. Additionally, there is interest in the development of strategies to mitigate enteric methane (CH₄) emissions from beef cattle as this potent greenhouse gas contributes significantly to climate change (FAO, 2010).

Humic substances (HS) are geological deposits made of a complex mixture of acids which arise from the natural decomposition of plant and animal material by soil microorganisms (McMurphy et al., 2009). Humic and fulvic acids are the major extractable components of soil humates and are predominantly used to improve soil fertility and enhance nutrient uptake by plants (Rajendiran et al., 2016). It has been proposed that HS act as natural antibiotics, resulting in increased growth performance in cattle and goats (Cusack, 2008; Degirmencioglu, 2012). They have antimicrobial (Váradýová et al., 2009), absorptive, and detoxifying properties, which in soils have been described to promote microbial growth (Islam, 2005). It has been proposed that a similar response may occur within the rumen, which could potentially enhance fermentation and digestibility of nutrients through increased microbial activity (Hayirli et al., 2005).

HS are known to exhibit a high affinity for nitrogen (N), a property that has been postulated to improve rumen microbial synthesis and decrease N excretion and CH₄ emissions into the environment (McMurphy et al., 2011). However, very few studies have attempted to test this hypothesis and those that did were not well designed or yielded

inconsistent results while measuring a limited number of response variables (Agazzi et al., 2007; Cusack, 2008; McMurphy et al., 2009; Váradýová et al., 2009; McMurphy et al., 2011; Degirmencioglu, 2012). The objective of this study was to investigate the effect of increasing concentrations of HS on rumen fermentation, nutrient digestibility, CH₄ production, and the rumen microbial population of beef cattle.

METHODS AND MATERIALS

The study was conducted at the Agriculture and Agri-Food Canada Research and Development Center in Lethbridge, Alberta, Canada. The animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, 2009), and the study was reviewed and approved by the institutional Animal Care Committee at the center.

Experimental Design

This experiment was designed as a replicated 4 × 4 Latin square design with 8 heifers (4/square), four 28-d periods, and four dietary treatments. Heifers were adapted to diets for the first 14 d of each period with the remaining 14 d used for measurements and sample collection.

Eight ruminally cannulated Angus × Hereford crossbred heifers (758 ± 40.7 kg initial BW) were blocked by weight and randomly assigned to a treatment sequence at the beginning of the experiment. Heifers were offered a basal diet consisting of 60% barley silage, 35% dry-rolled barley grain, and 5% vitamin and mineral supplement (DM basis; Table 1) with either 0- (control), 100-, 200-, or 300-mg HS/kg live BW (Canadian Humalite International Inc., Edmonton, AB, Canada). The HS consisted of

Table 1. Ingredient and chemical composition of the total mixed ration fed to heifers to investigate the effects of humic substances on rumen fermentation, nutrient digestibility, methane production, and the rumen microbiota

Component	TMR	Barley silage	Barley grain	Mineral supplement	Humic substance ¹
Ingredients, % DM		60.0	35.0	5.0	
Composition, % DM					
DM	53.0	43.3	92.5	94.4	75.7
OM	94.0	93.6	98.0	69.9	75.4
CP	13.4	13.0	14.2	17.5	7.10
NDF	30.8	41.7	25.3	30.4	
ADF	14.2	22.4	3.87	4.20	
Starch	38.4	28.3	62.4	35.1	
GE, Mcal/kg	5.09	5.64	4.83	3.32	

¹Humic substance was manufactured by Canadian Humalite International Inc., Edmonton, AB, Canada; 8.64 g/kg Ca, 1.23 g/kg Mg, 2.13 g/kg Fe, 0.34 g/kg K, 1.41 g/kg Na, 51.3 g/kg S, 0.14 g/kg Mn, 0.34 g/kg K, 0.31 g/kg Ti, 5270 mg/kg Al, 1.47 mg/kg As, 0.165 mg/kg Cd, and 11.6 mg/kg Pb.

50.7% humic and 4.4% fulvic acids (wet wt basis) as assessed using methods of the Humic Products Trade Association (Lamar *et al.*, 2014). Dietary concentrations of HS were selected based on an *in vitro* batch culture in which the same HS additive decreased CH₄ production and increased DM disappearance at 30 g/kg substrate DM (Sheng *et al.*, 2018). The basal diet was formulated to meet the nutrient requirements of beef heifers according to NASEM (2016). Heifers were housed in tie stalls and given daily exercise in an open dry lot, except during the time of digestibility and CH₄ measurements.

Feed Sampling and Intake

Heifers were restrictively fed a total mixed ration (TMR) as (NASEM, 2016) once daily at 0830 h. The HS was top dressed onto the ration at feeding and manually mixed into the diet. Samples of barley silage and TMR were sampled daily, pooled by week, and subsequently dried at 55 °C for 72 h. Subsamples were frozen at -20 °C until further chemical analysis. The diets were adjusted if the silage DM deviated more than 3 units from the average.

Rumen Fermentation

Rumen samples (300 g) from each heifer were collected on day 15 at 0, 3, 6, and 12 h after feeding from 4 sites (reticulum, ventral, caudal, and dorsal-ventral sac) within the rumen and pooled. Pooled samples of rumen contents (40 g) for microbial profiling were flash-frozen in liquid N and stored at -80 °C. Additional rumen samples were squeezed through 2 layers of PECAP nylon (pore size 355 µm; Sefar Canada Inc., Ville St. Laurent, Canada), pH of the filtrate was determined, and two 2-mL subsamples were placed in microtubes prefilled with 0.4 mL of 25% (wt/vol) metaphosphoric acid and 0.4 mL of 1% (wt/vol) sulphuric acid for analysis of VFA and ammonia (NH₃)-N, respectively. Samples were stored at -20 °C until analyzed. Additional filtrate (5 mL) was stored in a scintillation vial containing 5 mL of methyl green-formalin-saline solution (Ogimoto and Imai, 1981) for enumeration of protozoa and kept at room temperature in the dark.

Blood Sampling

On day 22 of each period, blood samples were collected from heifers via the jugular vein immediately before and 6 h after feeding. Blood was collected in two 10-mL vacuum tubes, containing lithium

heparin for plasma urea N (PUN; Vacutainer; Becton Dickinson, Mississauga, Canada). Tubes for PUN were centrifuged at 2,000 × *g* for 20 min at 4 °C and plasma was transferred into microtubes and frozen at -20 °C until analyzed.

Apparent Total Tract Digestibility

Apparent total tract digestibility of nutrients was determined by housing heifers in individual collection tie stalls. Total urinary and fecal collection was conducted for four 24-h periods between days 18 and 22. The heifers were fitted with urinary indwelling balloon catheters (Bardex Lubricath Foley catheter, Bard Canada Inc., Oakville, ON, Canada) to avoid cross-contamination of urine and feces. Urine was collected in a closed collection container and preserved with 4 N H₂SO₄ to prevent the volatilization of NH₃. Feces were collected in a pan placed behind the heifers. A 2% subsample of urine and a 10% subsample of feces were taken each day and composited by heifer for each period. Urine subsamples were diluted with distilled water at a ratio of 1:5 and stored at -20 °C until analyzed. Samples of the pooled feces (500 g) were dried at 55 °C for 4 d to determine DM content.

Methane Measurements

On day 26 of each period, heifers were housed in 4 separate open-circuit respiratory chambers (4.4-m wide × 3.7-m deep × 3.9-m tall, 63.5-m³ vol, model C1330; Conviron Inc., Winnipeg, MB, Canada) over 3 d for the quantification of CH₄ and CO₂ production, and O₂ consumption. Squares were staggered by a week as only 4 chambers were available at one time. A detailed description of the methodology and emission calculations is provided by Beauchemin and McGinn (2006). Briefly, heifers were placed in individual tie stalls within chambers with free access to water. Chamber doors were opened for feeding and cleaning once daily. Samples of intake and exhaust air from each chamber were sampled sequentially, every 30 min, by pumping 1 liter/min (TD3LS7; Brailsford and Company, Rye, NY) through infrared gas analyzers for CH₄ (Ultramat 6; Siemens, Karlsruhe, Germany), CO₂ (LI-7000 CO₂/H₂O analyzer; LI-COR Biosciences, Lincoln, NE), and O₂ (FC-10 Oxygen analyzer; Sable Systems International, Las Vegas, NV) via a set of solenoids controlled by a data logger (CR23X; Campbell Scientific, Logan, UT). The difference in concentration and volume of gases from intake and exhaust air was used to calculate

CH₄ and CO₂ produced and O₂ consumed by each individual heifer.

Chambers were calibrated at the beginning and the end of the experiment by sequentially releasing 0, 0.2, and 0.4 liter/min of CH₄ and CO₂ into each chamber using a mass-flow meter (Omega Engineering, Stamford, CT). Slopes of the best fit calibration regressions (actual against calculated CH₄ emission) were used to correct for variability among chambers as described by [Beauchemin and McGinn \(2006\)](#). Variability in slopes across chambers was less than 5% and recovery rates ranged from 97% to 107%.

Ruminal pH was recorded from day 26 during the 3 d of CH₄ measurements using the LRCpH data logger system (Dascor, Escondido, CA; [Penner et al., 2006](#)). The pH logger was placed in the ventral sac of the rumen 2 h before entry into the open-circuit respiratory chambers. Rumen pH was recorded in 1-min intervals and loggers were standardized in pH 4 and 7 before and after rumen measurements.

Chemical Analysis

Feed and fecal samples were oven dried at 55 °C and subsequently ground through a 1-mm screen (Standard model 4 Wiley mill; Arthur H. Thomas, Philadelphia, PA). Samples were then analyzed for analytical DM ([AOAC, 2005](#); method 930.15), OM (method 942.05), ash (method 942.05), NDF, and ADF. Ash content was determined by combustion of samples in a muffle furnace at 550 °C for 5 h. Samples were analyzed sequentially for NDF ([Mertens, 2002](#)) and ADF ([AOAC, 2005](#); method 973.18) with modifications for using a fiber analyzer (F57 Fiber Filter Bags, 200 Fiber Analyzer, ANKOM Technology; [Vogel et al., 1999](#)), with heat-stable α amylase (Termamyl 120, Sigma-Aldrich, St. Louis, MO) and sodium sulfite included in the NDF procedure which was expressed exclusive of residual ash.

Subsamples of dried feed and feces were further ground in a ball mill (Mixer Mill MM2000, Retsch, Haan, Germany) for determination of starch and N content. For urinary N, 150 μ L of diluted acidified urine was oven dried for 24 h. Nitrogen in feed, feces, and urine was quantified by flash combustion with gas chromatography and thermal conductivity detection (Carlo Erba Instruments, Milan, Italy; [AOAC, 2005](#); method 990.03) with CP calculated as N $\times$ 6.25. Starch was determined by hydrolyzing α -glucose polymers using a mixture of amyloglucosidase (Megazyme International Ltd., Wicklow,

Ireland) and 1,4- α -D-glucan glucanohydrolase (Brennfag Canada Inc., Toronto, ON, Canada) as described by [Herrera-Saldana et al. \(1990\)](#). Absorbance of samples was read on a Thermo Scientific Appliskan 1.437 (SkanIt Software 2.3 RE) microplate reader at a wavelength of 490 nm. Gross energy content of feed and fecal samples was determined using a bomb calorimeter (model E2k, CAL2k, Johannesburg, South Africa).

Protozoa were enumerated under a light microscope using a Levy-Hausser counting chamber (Hausser Scientific, Horsham, PA) with a 1-mm depth as described by [Dehority \(1993\)](#). Concentration of NH₃-N in rumen samples was analyzed by the phenol-hypochlorite method as described by [Broderick and Kang \(1980\)](#). Rumen VFA were determined by gas chromatography (5890A Series Plus II, Hewlett Packard Co., Palo Alto, CA). The chromatograph was equipped with a 30-m Zebron free fatty acid phase fused silica capillary, 0.32-mm i.d., and 1.0- μ m film thickness (Phenomenex, Torrance, CA). Concentration of PUN was determined using a microsegmented flow analyzer (model Astoria2; Astoria Pacific Inc., Clackamas, OR).

Uric acid in urine samples was determined by a colorimetric procedure using a commercial kit (MAK077, Sigma-Aldrich Co., St. Louis, MO) and allantoin by a colorimetric method as described by [Young and Conway \(1941\)](#).

Rumen Bacteria and Archaeal Diversity

For DNA extraction, frozen samples were lyophilized and ground using a coffee grinder. The DNA was extracted using repeated bead beating plus column (RBB + C; [Yu and Morrison, 2004](#)).

Sequencing was performed at McGill University and Génome Québec Innovation Center, Montréal, Canada using the Illumina MiSeq Reagent Kit v2 (500 cycle) following the manufacturer's guidelines. The primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') targeting the V4 region of the 16S rRNA gene were used to examine both Bacterial and Archaeal diversity. A 33 cycle PCR using 1 μ L of a 1 in 10 dilution of genomic DNA and the FastStart High Fidelity PCR System (Roche, Montreal, PQ) was conducted with the following conditions: 94 °C for 2 min, followed by 33 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, with a final elongation step at 72 °C for 7 min. Fluidigm Corporation (San Francisco, CA) barcodes were incorporated in a

second PCR reaction using the FastStart High Fidelity PCR System under the following conditions: 95 °C for 10 min, followed by 15 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min, followed by a final elongation step at 72 °C for 3 min. After amplification, PCR products were assessed in a 2% agarose gel to determine the success of amplification. All samples were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Carlsbad, CA) and were pooled in equal proportions. Pooled samples were then purified using calibrated Ampure XP beads (Beckman Coulter, Mississauga, ON). The pooled samples (library) were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Carlsbad, CA) and the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems, Wilmington, MA). Average fragment size was determined using a LabChip GX (PerkinElmer, Waltham, MA, USA) instrument.

Sequence and quality analyses of the raw fastq files from the MiSeq were checked with the FastQC program (Andrews, 2010). Trimmomatic v0.33 (Bolger et al., 2014) was used to trim the raw reads as well as to remove ambiguous and low quality reads. Reads with average quality score of <20 over a 4-bp sliding window, and reads with lengths shorter than 36 bp were removed. The paired-end reads were merged using PEAR v0.9.8 using default options (Zhang et al., 2014) and unassembled reads were discarded. High-quality sequence reads from the various samples were then combined into a single dataset and subsequent analysis was carried out with QIIME V1.9.0 (Caporaso et al., 2010a). Detection/removal of chimeric sequences and clustering of Operational Taxonomic Units (OTU) at 97% id was carried out using USEARCH61 (Edgar, 2010) using an open reference OTU picking approach. Rare, low abundance OTU (<0.005% abundance) were not considered and were removed from the OTU table. Taxonomy was assigned based on the Greengenes 13.8 reference sequence dataset (McDonald et al., 2012) using UCLUST (Edgar, 2010). The OTU were aligned using PyNAST (Caporaso et al. 2010b) against the Greengenes 13.8 aligned reference dataset (McDonald et al., 2012). Aligned OTU were used to construct a phylogenetic tree with FastTree (Price et al., 2010). Sequences have been deposited to the Small Reads Archive (NCBI) with accession number SRP129886.

Microbial diversity within (α -diversity) and between samples (β -diversity) was assessed using

Qiime. α -Diversity measures for richness (Chao1), phylogenetic diversity (branch length based diversity), evenness (Simpsons), number of observed OTU, and taxonomic abundance were evaluated. Sequences were subsampled to the lowest number of sequences found in all samples to ensure that α - and β -diversity analysis used the same number of sequences per sample. β -Diversity analysis was carried out using weighted and unweighted UniFrac (Lozupone, 2011).

Calculations and Statistical Analysis

Microbial N synthesis was estimated using concentrations of allantoin and uric acid in urine as described by Chen and Gomes (1992). Microbial purine derivatives (PD) absorbed (mmol/d) was calculated as follows:

$$\text{Microbial PD absorbed} = \left(\frac{\text{Total PD excretion} -}{0.385 \times BW^{0.75}} \right) / 0.85,$$

where total PD is the sum of uric acid (mmol/d) and allantoin (mmol/d) excreted and 0.85 is the efficiency of absorption of PD. Microbial N (g N/d) flow was calculated as follows:

$$\text{Microbial N flow} = (\text{purine absorption} \times 70) / (0.116 \times 0.83 \times 1000),$$

where purine adsorption is in mmol/d; 70 is the N content of purines (mg N/mmol), 0.116 is the ratio of purine N:total N for mixed rumen microbes, and 0.83 is the digestibility of microbial purines (Chen and Gomes, 1992).

Heat production was calculated using the average measurements of O₂ consumed and CH₄ and CO₂ produced over the 3 d and applied to the following equation (Brouwer, 1965):

$$\begin{aligned} \text{HP (kcal / d)} &= 3.886 \times \text{O}_2 (\text{L / d}) + 1.200 \times \text{CO}_2 \\ &(\text{L / d}) - 1.431 \times \text{urinary} \\ &\text{N (g / d)} - 0.518 \times \text{CH}_4 (\text{L / d}). \end{aligned}$$

Data were analyzed using the mixed model procedure of SAS (SAS Institute Inc., Cary, NC). The univariate procedure in SAS was used to test for normal distribution. Data were analyzed with heifer as experimental unit for all variables. Concentration of HS (0, 100, 200, and 300 mg HS/kg BW) was included as fixed effects and random effects were square, heifer nested within square, and period nested within square. For

rumen fermentation variables, sampling time was treated as a repeated measure and protozoa numbers were $\log_{10}$ transformed before statistical analysis. Continuous ruminal pH data of each heifer were summarized for daily average, minimum, maximum, and standard deviation using SAS. Data obtained from the chambers were averaged by time point (every 30 min), heifer, and period for statistical analysis. For pH measurements and CH_4 production, day was treated as a repeated measure. The minimum values of Akaike's information criterion were used to select the covariance structure. Contrast statements were used to test the linear, quadratic, and control vs. HS effects, across all treatments. False discovery rate (FDR)-corrected P values were calculated using Tukey's test. Differences between means were declared significant at $P < 0.05$.

Principal coordinate plots based on unweighted and weighted unifac distance matrices were generated using the script `principal_coordinates.py` within QIIME 1.9.1. Correlation analysis was used to test the relationship among diet digestibility parameters, rumen fermentation characteristics, and relative taxa abundances of archaea and bacterial populations using PROC CORR procedure

of SAS. The resulting correlation matrix was visualized in a heatmap format generated by the `corrplot` package of RStudio (Corrplot: visualization of a correlation matrix; RStudio Version 1.1.383 2017).

RESULTS

Dry matter intake was similar ($P = 0.47$) across treatments (Table 2). Total VFA and their individual concentrations were not affected ($P \geq 0.29$) by the addition of HS. The concentration of $\text{NH}_3\text{-N}$ and total protozoa count responded quadratically ($P = 0.03$) to increasing concentrations of HS. The minimum, mean, max, and standard deviation of ruminal pH were not affected by HS.

Although apparent total tract digestibility of DM was not affected ($P \geq 0.77$), digestibility of CP linearly increased ($P = 0.04$) with increasing dietary HS concentration (Table 3). Starch digestibility exhibited a quadratic response ($P = 0.03$) to the inclusion of HS.

There was a linear tendency ($P = 0.09$) for decreased total N excretion with HS when compared with control (Table 4). Fecal N excretion (g/d) was linearly decreased ($P < 0.04$) with increasing

Table 2. Dry matter intake, ruminal fermentation parameters, protozoa counts, and rumen pH in beef heifers fed a barley silage-based diet containing increasing concentrations of humic substances (HS; $n = 8$ per treatment)

Item	Treatment ¹						P value ²	
	Control	H100	H200	H300	SEM	L	Q	Control vs. HS
DMI, kg/d	7.90	7.97	8.03	8.08	0.181	0.47	0.96	0.55
Total VFA, mM ³	116.6	114.0	117.3	115.7	5.78	0.98	0.93	0.89
VFA, mM ³								
Acetate (A)	79.6	78.8	81.1	79.1	4.21	0.97	0.87	0.99
Propionate (P)	20.3	17.9	19.0	19.9	1.56	0.98	0.20	0.55
Butyrate	9.90	11.0	10.8	10.3	0.63	0.79	0.21	0.30
Isobutyrate	1.33	1.26	1.39	1.28	0.102	0.95	0.85	0.32
Valerate	1.73	1.64	1.80	1.68	0.102	0.97	0.88	0.86
Isovalerate	2.38	2.20	2.58	2.15	0.183	0.72	0.51	0.75
Caproate	0.75	0.64	0.71	0.69	0.064	0.66	0.43	0.29
A:P ratio	3.92	4.40	4.27	3.97	0.251	0.76	0.32	0.37
$\text{NH}_3\text{-N}$, mM ³	5.71	6.54	6.37	5.41	1.221	0.54	0.03	0.91
Protozoa, $n \times 10^{5,3}$	4.23	5.45	6.25	3.78	0.952	0.89	0.03	0.32
Ruminal pH ⁴								
Min	5.47	5.41	5.52	5.58	0.131	0.46	0.67	0.82
Mean	6.30	6.22	6.27	6.21	0.139	0.73	0.94	0.69
Max	6.85	6.90	6.93	6.94	0.044	0.16	0.63	0.18
SD	0.38	0.38	0.42	0.37	0.043	0.89	0.57	0.76

¹Control: no HS; H100: 100 mg/kg BW of HS; H200: 200 mg/kg BW of HS; H300: 300 mg/kg BW of HS.

²L = linear effect; Q = quadratic effect; Control vs. HS = control vs. HS treatments.

³No. of observations = 32.

⁴Ruminal pH is given by continuous pH loggers over 3 d.

Table 3. Nutrient digestibility of a barley silage-based diet containing increasing concentrations of humic substances in beef heifers ($n = 8$ per treatment)

Item	Treatment ¹				SEM	P value ²		
	Control	H100	H200	H300		L	Q	Control vs. HS
Digestibility, %								
DM	68.1	68.0	68.0	67.7	1.55	0.77	0.95	0.82
OM	69.5	69.6	69.7	69.4	1.51	0.97	0.86	0.95
NDF	45.9	45.8	45.9	44.5	3.57	0.66	0.77	0.82
ADF	33.0	33.2	31.9	28.1	5.20	0.23	0.50	0.56
CP	62.1	64.7	64.6	65.0	2.08	0.04	0.21	0.02
Starch	94.1	94.6	95.6	93.7	0.70	1.00	0.03	0.32
GE	66.0	66.1	66.0	65.8	1.62	0.87	0.88	0.98

¹Control: no HS; H100: 100 mg/kg BW of HS; H200: 200 mg/kg BW of HS; H300: 300 mg/kg BW of HS.

²L = linear effect; Q = quadratic effect; Control vs. HS = control vs. HS treatments.

Table 4. Nitrogen intake and excretion, plasma urea nitrogen, and purine derivatives of beef heifers fed a barley silage-based diet containing increasing concentrations of humic substances ($n = 8$ per treatment)

Item	Treatment ¹				SEM	P value ²		
	Control	H100	H200	H300		L	Q	Control vs. HS
N intake, g/d	171.9	172.8	173.4	173.9	3.69	0.001	0.30	0.001
Fecal N excretion, g/d	63.7	59.4	59.3	59.4	2.35	0.04	0.10	0.01
Urine N excretion, g/d	85.4	82.2	83.8	83.7	3.45	0.76	0.55	0.49
Total N excretion, g/d	149.1	141.6	143.1	143.1	4.01	0.25	0.22	0.09
Total N retention								
Retained N ³ , g/d	22.5	31.2	30.3	30.8	4.72	0.10	0.19	0.04
Retained N, % N intake	12.9	17.9	17.2	17.7	2.54	0.10	0.21	0.04
Retained N, g/kg BW ^{0.75}	0.15	0.21	0.20	0.21	0.029	0.08	0.17	0.03
Plasma urea N, mg/dL	11.8	12.0	11.8	12.1	0.78	0.88	0.99	0.86
Total PD ⁴ , mmol/d	142.6	139.0	142.3	140.8	6.41	0.93	0.85	0.76

¹Control: no HS; H100: 100 mg/kg BW of HS; H200: 200 mg/kg BW of HS; H300: 300 mg/kg BW of HS.

²L = linear effect; Q = quadratic effect; Control vs. HS = control vs. HS treatments.

³Intake = feces – urine.

⁴PD = purine derivatives.

concentrations of HS. Total N retention (g/d, g/g N intake, g/kg BW^{0.75}) was improved ($P < 0.04$) for HS when compared with the control. The excretion of total PD was not affected ($P = 0.93$) by HS. Energy balance, BW, and CH₄ production were also not affected (Tables 5 and 6).

Illumina sequencing generated a total of 4,779,689 high quality reads with a total of 4,322,703 read pairs assembled and the contigs were used to assess the impact of HS on the rumen microbiome. The number of contigs in samples ranged from 89,823 to 148,855. The OTU clustering resulted in a total of 2,154 unique OTU that were identified across all samples. Goods coverage for all samples was >0.99 and rarefaction curves reached an asymptote indicating that the depth of sequencing was sufficient to capture most of the microbial diversity. Sequences were randomly subsampled to select 89,823 reads from all samples for use in downstream analysis of α - and β -diversity.

The total number of reads; the number of unique reads; the number of observed OTU (97% identity cutoff); and measures of richness (Chao1), diversity (PD whole tree), and evenness (Simpsons) showed no difference ($P \geq 0.23$) among treatments (Table 7).

There were 18 bacterial phyla detected in the rumen samples (Figure 1). The microbiomes were all dominated by Bacteroidetes and Firmicutes with averages of $40.1\% \pm 0.9$ and $35.7\% \pm 0.9$ of the OTU being classified to these phyla. Classification at the phylum level was generally high, with only 0.3% of the OTU not being classified to this taxonomic level. Other abundant bacterial phyla (>1% abundance) included Fibrobacteres ($9.8\% \pm 0.03$), Spirochetes ($2.8\% \pm 0.1$), Proteobacteria ($2.5\% \pm 0.3$), and Verrucomicrobia ($1.5\% \pm 0.1$). Phyla with <1% abundance were Actinobacteria, Planctomycetes, Chloroflexi, Cyanobacteria,

Table 5. Dry matter intake, body weight, and methane emissions from beef heifers fed a barley silage-based diet containing increasing concentrations of humic substances ($n = 24$ per treatment)

Item	Treatment ¹				SEM	P value ²		
	Control	H100	H200	H300		L	Q	Control vs. HS
BW, kg	790	785	785	785	17.3	0.82	0.88	0.77
CH ₄								
g/d	255.0	255.5	252.7	249.9	21.28	0.58	0.87	0.77
g/kg DMI	32.3	31.8	31.3	30.7	2.28	0.60	0.99	0.69
g/kg DMD	47.6	46.9	46.0	45.1	3.12	0.55	0.97	0.66
g/kg BW	0.32	0.33	0.32	0.32	0.028	0.89	0.84	0.98

¹Control: no HS; H100: 100 mg/kg BW of HS; H200: 200 mg/kg BW of HS; H300: 300 mg/kg BW of HS.

²L = linear effect; Q = quadratic effect; Control vs. HS = control vs. HS treatments.

³Methane emissions were determined over 3 d.

Table 6. Energy balance of beef heifers fed a barley silage-based diet containing increasing concentrations of humic substances ($n = 8$ per treatment)

Item	Treatment ¹				SEM	P value ²		
	Control	H100	H200	H300		L	Q	Control vs. HS
Energy components, Mcal/d								
GEI ³	41.8	41.8	41.8	41.8	0.64	0.54	0.16	0.22
DE, ⁴ Mcal/ kg DM	3.58	3.57	3.57	3.51	0.201	0.59	0.78	0.76
Methane	3.09	3.09	3.06	2.95	0.255	0.21	0.45	0.51
HP ⁵	14.2	14.5	14.5	14.0	0.89	0.77	0.08	0.35
GEI, %								
DE	67.5	66.8	67.3	66.1	1.71	0.30	0.74	0.42
Methane	7.34	7.34	7.31	7.20	0.556	0.61	0.78	0.80
ME ⁶	55.7	55.2	55.8	54.3	1.94	0.29	0.52	0.47
HP	33.9	34.6	34.8	33.7	2.26	0.81	0.10	0.34
RE ⁷	21.8	20.6	22.2	20.6	3.38	0.54	0.82	0.41

¹Control: no HS; H100: 100 mg/kg BW of HS; H200: 200 mg/kg BW of HS; H300: 300 mg/kg BW of HS.

²L = linear effect; Q = quadratic effect; Control vs. HS = control vs. HS treatments.

³GEI = gross energy intake = GE content (Mcal/kg DM) $\times$ DMI (kg/d).

⁴Digestible energy = EI (Mcal/d) – fecal energy (Mcal/d).

⁵Heat production as calculated by Brouwer (1965).

⁶Metabolizable energy = 100 – fecal energy (%) – urinary energy (%) – methane energy (%).

⁷Retained energy = ME(%) – HP(%).

Elysitimicrobia, Lentisphaerae, SR1, Synergistes, Tenericutes, LD1, and WPS-2.

Of the 18 detected phyla, 7 showed differences in abundance as a result of addition of HS to the diet. Firmicutes ($P = 0.06$), Euryarchaeota ($P = 0.04$), Planctomycetes ($P = 0.08$), and Synergistetes ($P < 0.01$) increased in abundance, whereas Fibrobacteres ($P = 0.06$) and Proteobacteria ($P = 0.05$) decreased in abundance with the addition of HS to the diet. There was a quadratic effect of HS on the abundance of Proteobacteria ($P = 0.05$), Actinobacteria ($P < 0.06$), and Planctomycetes ($P < 0.08$).

A total of 64 bacterial families were detected in all rumen samples (Figure 2). The 10 most abundant were Prevotellaceae ($25.1\% \pm 1.3$), Unclassified Clostridiales ($11.3\% \pm 0.4$), Fibrobacteraceae (10.8%

± 0.6), Ruminococcaceae ($8.34\% \pm 0.4$), Unclassified Bacteroidales (8.14 ± 0.5), Lachnospiraceae ($6.63\% \pm 0.25$), Veillonellaceae ($5.53\% \pm 0.15$), Methanobacteriaceae ($3.38\% \pm 0.19$), Spirochaetaceae ($2.95\% \pm 0.11$), and Paraprevotellaceae ($2.66\% \pm 0.12$). There was a linear increase in the level of Christensenellaceae ($P < 0.03$), unclassified Clostridiales ($P < 0.01$), RFP12 ($P < 0.05$), and unclassified Tenericutes RF39 ($P < 0.001$) as a result of HS. The abundance of Spirochaetaceae linearly decreased ($P < 0.05$) with increasing HS.

An average of $3.6\% \pm 0.2$ of the reads were classified as Archaea, predominantly Methanobrevibacter (3.4%) with minimal OTU within the genus VadinCA11 (0.2%) belonging to the Class Thermoplasmata. At the phylum level, HS had an effect ($P < 0.04$) on the abundance

Table 7. α -Diversity indices of bacterial communities in the rumen of beef heifers fed a barley silage-based diet containing increasing concentrations of humic substances ($n = 8$ per treatment)

Item	Treatment ¹				SEM	<i>P</i> value ²		
	Control	H100	H200	H300		L	Q	Control vs. HS
Reads (Total)	109695	110853	109594	117548	4740.1	0.40	0.83	0.39
Reads (Unique)	1858	1926	1865	1829	49.1	0.41	0.20	0.76
Observed OTU	1837	1902	1844	1800	49.4	0.37	0.18	0.81
Chao1	1870	1961	1913	1880	43.3	0.90	0.11	0.29
Simpsons Evenness	0.06	0.08	0.07	0.07	0.011	0.60	0.97	0.10
Phylogenetic Diversity	129	135	131	129	2.9	0.82	0.12	0.32

¹Control: no HS; H100: 100 mg/kg BW of HS; H200: 200 mg/kg BW of HS; H300: 300 mg/kg BW of HS.

²L = linear effect; Q = quadratic effect; Control vs. HS = control vs. HS treatments.

Figure 1. Taxonomic composition of rumen samples classified at the phylum level collected from beef heifers fed a barley silage-based diet containing increasing concentrations of humic substances ($n = 8$ per treatment). Control: no HS; H100: 100 mg/kg BW of HS; H200: 200 mg/kg BW of HS; H300: 300 mg/kg BW of HS.

of Euryarchaeota with a linear trend ($P = 0.08$) for higher abundance in the presence of HS. The abundance of Methanobacteriaceae also increased ($P < 0.05$) with HS.

With the exception of heifer 8, weighted and unweighted unifracs calculations show that the microbial communities in all heifers did not change significantly as a result of HS addition or period (Figures 2 and 3, respectively). Microbial community structures generally tended to cluster on the basis of individual heifer (Figure 3).

A Pearson correlation matrix was created to assess relationships between relative abundance

of taxa and physiological responses in heifers (Figure 4). Parameters in the correlation matrix were selected based on whether they responded significantly to HS. The digestibility of NDF was positively correlated with Firmicutes (Pearson correlation coefficient (r) = 0.54, $P < 0.01$), Verrucomicrobia ($r = 0.45$, $P < 0.01$), Synergistetes ($r = 0.36$, $P < 0.05$), Verrucomicrobia RFP12 ($r = 0.53$, $P < 0.01$), and Dethiosulfovibrionaceae ($r = 0.45$, $P < 0.01$), and negatively associated with Bacteroidetes ($r = -0.47$, $P < 0.01$) and Proteobacteria ($r = -0.46$, $P < 0.01$). The digestibility of CP was positively correlated with Firmicutes ($r = 0.37$, $P < 0.05$) and Spirochaetaceae

Figure 2. Taxonomic composition of rumen samples classified at the family level collected from beef heifers fed barley silage based-diet containing increasing concentrations of humic substances ($n = 8$ per treatment). Control: no HS; H100: 100 mg/kg BW of HS; H200: 200 mg/kg BW of HS; H300: 300 mg/kg BW of HS.

($r = 0.40$, $P < 0.05$) and negatively correlated with Bacteroidetes ($r = -0.49$, $P < 0.01$). Ammonia-N was positively correlated with Firmicutes ($r = 0.40$, $P < 0.05$), Verrucomicrobia ($r = 0.45$, $P < 0.05$), Verrucomicrobia RFP12 ($r = 0.37$, $P < 0.05$), and Dethiosulfovibrionaceae ($r = 0.38$, $P < 0.05$) and negatively correlated with Bacteroidetes ($r = -0.39$, $P < 0.05$). There were correlations between rumen pH and Bacteroidetes ($r = -0.56$, $P < 0.001$), Firmicutes ($r = 0.56$, $P < 0.001$), Proteobacteria ($r = -0.37$, $P < 0.05$), Verrucomicrobia ($r = 0.58$, $P < 0.001$), Synergistetes ($r = 0.64$, $P < 0.001$), Verrucomicrobia RFP12 ($r = 0.55$, $P < 0.01$), and Dethiosulfovibrionaceae ($r = 0.61$, $P < 0.001$). Methane production was negatively correlated with Bacteroidetes ($r = -0.47$, $P < 0.01$) and Proteobacteria ($r = -0.50$, $P < 0.01$) and positively associated with Firmicutes ($r = 0.39$, $P < 0.05$), Euryarchaeota ($r = 0.37$, $P < 0.05$), Synergistetes ($r = 0.52$, $P < 0.01$), Verrucomicrobia RFP12 ($r = 0.43$, $P < 0.05$), and Dethiosulfovibrionaceae ($r = 0.53$, $P < 0.01$).

DISCUSSION

A number of studies have examined the value of HS as a feed additive for ruminants (Agazzi

et al., 2007; Cusack, 2008; McMurphy et al., 2009; Váradyová et al., 2009; McMurphy et al., 2011; Degirmencioglu, 2012). However, these studies did not examine the comprehensive effect of HS on rumen metabolism, and most of these studies were limited in scope (e.g., small sample size, poor experimental design, and limited measured parameters). It is also difficult to compare responses observed from previous studies where the chemical composition or concentration of humic and fulvic acids is not reported. The current experiment is the first comprehensive examination of the effects of HS in cattle, utilizing open-circuit respiratory chambers to quantify CH_4 emissions, and 16S rRNA sequencing to investigate the effect of HS on rumen bacterial and archaeal populations.

The quadratic response of protozoa numbers and $\text{NH}_3\text{-N}$ concentration to HS dose is consistent with the concept that rumen protozoa engulf rumen bacteria that use $\text{NH}_3\text{-N}$ to help meet their N requirements (Koenig et al., 2000; Bach et al., 2005). Thus, increased protozoa numbers may have led to less ruminal NH_3 incorporated into microbial protein and increased NH_3 concentrations. Protozoa also contribute to the degradation of feed protein releasing $\text{NH}_3\text{-N}$ as a result of deamination

Figure 3. Principal coordinate analysis of unweighted (top) and weighted unifracs distances clustered according to (A) concentration of humic substance in the diet (control – Red, 100 mg – blue, 200 mg – orange, 300 mg – green), (B) period (P1 – red, P2 – blue, P3 – orange, P4 – green), and (C) heifer (2 – Red, 6 – Blue, 8 – Orange, 9 – Green, 10 – purple, 11 – Yellow, 12 – Cyan, Pink – 21) ($n = 8$ per treatment).

Figure 4. Pearson Correlation between production variables and relative taxa abundance. Significant correlations are indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Strong correlations are indicated by large circles and darker colors, and weaker correlations indicated by smaller circles with weak colors. The scale colors denote whether the correlation is positive (blue) or negative (red).

of amino acids. Humates have been proposed to act as a natural antibiotic (Degirmencioglu, 2012) with antimicrobial properties that may inhibit protozoa when high concentrations are added to the diet (Bell et al., 1997). Váradyová et al. (2009) found that when HS were added at 10 g/kg DM to a rumen stimulation (Rusitec) using sheep inoculum, $\text{NH}_3\text{-N}$ was reduced by 24.4% in a high-forage diet. Those results contrast with the results from the current study, in which, at a similar concentration, HS increased $\text{NH}_3\text{-N}$ concentration and the number of protozoa. Other in vitro experiments have reported similar decreases in $\text{NH}_3\text{-N}$ concentration with increasing addition of humates (Bell et al., 1997; Sheng et al., 2018). The present study is the first to report an effect of HS on $\text{NH}_3\text{-N}$ concentration and protozoa counts in vivo. Although there was an increase in protozoa counts for the H100 and H200 treatments and a decrease for the H300 treatment, these changes were not sufficient to alter other aspects of rumen metabolism (OM digestibility, PD excretion, total VFA production, and CH_4 production) that are often associated with fluctuation in protozoal populations in the rumen (Newbold et al., 2015).

Ammonia that is not utilized to support amino acid synthesis for microbial growth is absorbed across the rumen epithelium and into blood where it may be excreted directly in the urine, used in the transamination to form glutamine, or converted to urea in the liver and excreted in the urine (Bach et al., 2005). When ruminal $\text{NH}_3\text{-N}$ concentration is high, the rate of urea-N recycling to the rumen decreases and N excretion in the urine increases (Storm et al., 2011). However, in the present study, there was no change in PUN or urinary N excretion as a result of inclusion of HS in the diet (Bach et al., 2005).

Although addition of HS increased N intake, HS tended to decrease total N excretion, mainly as a result of a reduction in fecal N excretion leading to an increase in apparent CP digestibility. A similar response was observed when pigs were supplemented with up to 4 g/d of HS and fecal N excretion was decreased and apparent total N digestion was increased (Ponce et al., 2016). Shi et al. (2001) also found evidence of the N binding capacity of HS when black humates were added to a 1950-g mixture of soil, feces, and urine at 1.7% (9000 kg/ha application equivalent). Soil NH_3 emissions were decreased by 39.8% compared with control (Shi et al., 2001). There are several mechanisms by which additives to soil can act to reduce NH_3 emissions including effects of pH, cation-anion exchange,

inhibition of enzymatic breakdown of nitrogenous compounds, and the carbon:N ratio (Shi et al., 2001). Dong et al. (2009) proposed that humic acids can stabilize extracellular enzymes including urease, a key enzyme that converts urea to NH_3 . They reported that soils amended with 10-mg urea/g soil and 1 mg of lignite humic acid/g soil delayed the release of NH_4 during urea hydrolysis. Humic and fulvic portions of HS can act as strong chelators (Mackowiak et al., 2001) and may be responsible for binding ions and minerals, possibly converting them to a chemical state that is more readily absorbed by cells and organisms. Trace elements within HS may also act as cofactors in N digestibility, increasing the activity of enzymes for increased digestion and utilization of nutrients (Hayirli et al., 2005). Although there are several possible modes of action of HS on microbial populations, which have been examined mostly in soils, it is uncertain if these substances are metabolized in the rumen and how this might affect their mode of action.

There were no significant differences in urinary N excretion in response to HS however, N retention (g/d, g N intake, and g/kg $\text{BW}^{0.75}$) increased for HS when compared with the control. Although animals were fed at maintenance, heifers still gained an average of 51.1 kg over the duration of the experiment, resulting in positive N retention. HS can incorporate N into their structure either directly via chemical means or indirectly through microbial activity and subsequent decomposition of microbial biomass (Dong et al., 2009). Chemical reactions between NH_3 and HS have been observed and can be related to aromatic amines, indoles, pyrroles, and other functional structures within HS (Clinton et al., 1995). The weak acids and organic colloids that make up humates can adsorb metal ions by desorption (Ugapo and Pickering, 1985), theoretically binding to substances or small particles in the rumen and releasing them for further digestion or absorption in the small intestine. These mechanisms of action may be supported by other studies that have found inclusion of a humic and fulvic acid complex at 20 g/steer/d, half of that provided by the H100 treatment, increased weight gain, and improved the feed efficiency of feedlot cattle (Cusack et al., 2008). Similarly, Agazzi et al. (2007) indicated that ADG was increased in goats, which were orally supplemented with a humate solution at up to 30 mL/d at 4 to 8 wk of age. McMurphy et al. (2009) included up to 1.5% DM of a humic and fulvic complex in the diets of crossbred steers and concluded that it displayed ionophore-like properties with similar responses in BW, ADG, and

gain-to-feed ratio to that achieved through supplementation with the ionophore, monensin. However, [McMurphy et al. \(2009\)](#) did not utilize a negative control and a subsequent experiment conducted by the same research group including up to 15-g/kg diet DM of humic acids in a high-concentrate beef finishing diet found no effects on rumen fermentation ([McMurphy et al., 2011](#)). The concentrations used in both studies by [McMurphy et al. \(2009, 2011\)](#) are comparable to the 200-mg/kg BW treatment used in the current study. With regards to N excretion and retention, there seemed to be no benefit from including greater concentrations of HS as these parameters were similar among HS treatments.

Firmicutes are mainly comprised of Gram-positive, low-G+C-content bacteria ([Bevans et al., 2005](#)), and an increased ratio of Firmicutes to Bacteroidetes has been considered as a biomarker for the metabolic potential of the gut microbiome ([Derakhshani et al., 2017](#)). In the current study, the digestibility of CP was negatively correlated with Bacteroidetes and positively correlated with Firmicutes. The Firmicutes phyla and families within this phylum (Christensenellaceae, Lachnospiraceae, and Unclassified Clostridiales) were increased with HS. The Spirochaetaceae were the only other family that exhibited a change as a result HS and a positive correlation with CP digestibility. Spirochaetaceae are spherical, anaerobic bacteria and have been suggested to have a role in fiber degradation in cattle ([McCann et al., 2014](#)), as well as N_2 fixation in termites ([Lilburn et al., 2001](#)). Although HS altered the abundance of these bacteria, it is unclear whether these changes were related to the specific changes observed in rumen fermentation and metabolism.

HS have been shown to act as electron acceptors for a large variety of microorganisms capable of extracellular electron transfer, including methanogens ([Martinez et al., 2013](#)). The redox capabilities of HS have been attributed to a variety of functional structures like quinone, phenolic hydroxyl, and N- and sulfur-containing molecules ([Aeschbacher et al., 2010](#)). The redox potential of these chemical structures has shown to lower CH_4 production from soil microorganisms as [Tan et al. \(2018\)](#) found that HS suppressed CH_4 production in anoxic environments when included in batch incubations at 60 mg/liter. However, there are inconsistencies in the effects of HS in soils as other authors found that when the same HS were used in anoxic paddy soils at 0.5mM, they actually facilitated methanogenesis ([Zhou et al. 2014](#)).

Using rumen fluid, evidence of HS lowering CH_4 was seen by [Sheng et al. \(2018\)](#) who included HS at up to 30 g/kg of dietary DM and found that CH_4 production was decreased in vitro.

The lack of change in CH_4 production in vivo is supported by the relative abundances of archaeal and bacterial populations. Euryarchaeota are CH_4 -producing archaea and their abundance was positively correlated with CH_4 production, whereas Proteobacteria, associated with the utilization of CH_4 ([Mitsumori et al., 2002](#)), decreased with the addition of HS. Additionally, the phylum Synergistetes was increased by HS compared with the control. This phylum is known for its ability to degrade amino acids and pyruvate ([Han et al., 2015](#)); however, it was shown to have a positive correlation with Euryarchaeota abundance and CH_4 production as well as a negative correlation with Proteobacteria. Our findings are in agreement with [Wallace et al. \(2015\)](#) who demonstrated that Proteobacteria were 0.24 times less abundant, and Synergistetes were 1.95 times more abundant in cattle that were greater emitters of CH_4 , suggesting that members of these phyla play a role in CH_4 metabolism.

In conclusion, the addition of HS to the diet of beef heifers resulted in a favorable increase in the retention of N, with increased NH_3 -N and protozoa counts at low to moderate doses of HS. HS favorably increased the digestibility of CP and the retention of N, and decreased fecal N excretion. The addition of HS to the diet had no effect on CH_4 production and the microbiome was altered in a manner that was consistent with the lack of change in CH_4 production. Further study should assess the effects of HS additive on growth performance in feedlot cattle.

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