Effect of engineered biocarbon on rumen fermentation, microbial protein synthesis, and methane production in an artificial rumen (RUSITEC) fed a high forage diet[1]

Atef M. Saleem,[*,†] Gabriel O. Ribeiro Jr,[*] Wenzhu Z. Yang,[*] Tao Ran,[*,‡] Karen A. Beauchemin,[*] Emma J. McGeough,[#] Kim H. Ominski,[#] Erasmus K. Okine,[§] and Tim A. McAllister[*,2]

[*]Lethbridge Research and Development Centre, Agriculture and Agri-Food Canada, Lethbridge, AB, T1J 4B, Canada; [†]Department of Animal and Poultry Production, Faculty of Agriculture, South Valley University, Qena 83523, Egypt; [‡]Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, Hunan 410125, P.R. China; [#]Department of Animal Science, University of Manitoba, Winnipeg, R3T 2N2, Canada; and [§]Department of Biological Sciences, University of Lethbridge, Lethbridge, Alberta, T1K 3M4, Canada

ABSTRACT: The objective of this study was to investigate the effects of adding engineered biocarbon to a high-forage diet on ruminal fermentation, nutrient digestion, and enteric methane (CH_4) production in a semi-continuous culture artificial rumen system (**RUSITEC**). The experiment was a completely randomized block design with four treatments assigned to sixteen fermentation vessels (four/treatment) in two RUSITEC apparatuses. The basal diet consisted of 60% barley silage, 27% barley grain, 10% canola meal, and 3% supplement (DM basis) with biocarbon added at 0, 0.5, 1, and 2% of substrate DM. The study period was 17 d, with a 10-d adaptation and 7-d sample collection period. Increasing biocarbon linearly increased ($P < 0.05$) disappearance of DM, OM, CP, ADF and NDF. Compared to control, increasing biocarbon enhanced ($P < 0.01$) production of total VFA, acetate, propionate, branch-chained VFAs, and tended to increase ($P = 0.06$) NH_3-N. Microbial protein synthesis linearly increased ($P = 0.01$) with increasing biocarbon. Addition of biocarbon reduced overall CH_4 production compared with the control ($P \leq 0.05$). There were no differences ($P > 0.05$) in production of total gas, large or small peptides, or in the number of protozoa as a result of addition of biocarbon to the diet. Addition of biocarbon to a forage diet increased DM digestibility by up to 2%, while lowering enteric CH_4 production and enhancing microbial protein synthesis in in vitro semi-continuous culture fermenters.

Key words: biocarbon, biochar, methane, rumen fermentation, RUSITEC

J. Anim. Sci. 2018.96:3121–3130
doi: 10.1093/jas/sky204

INTRODUCTION

Improving forage utilization is critical for the profitability of all sectors of the beef cattle industry. Methane (CH_4) and carbon dioxide (CO_2) are natural byproducts of ruminant fermentation, with total gas production being primarily dependent on the microbial efficiency of feed conversion (Hansen et al., 2012). Methane has a global warming potential that is 28 times that of CO_2 (Myhre et al., 2013). Mitigation of CH_4 emissions in ruminants is seen as a key combatant to climate change as it accounts for 28% of global anthropogenic CH_4 emissions (US-EPA, 2011). Hence, the cattle industry has an increasing incentive to develop strategies to mitigate enteric CH_4 production. Dietary manipulation

[1]This project was supported by the Agriculture Greenhouse Gases Program of Agriculture and Agri-Food Canada. Authors wish to acknowledge technical staff at the Lethbridge Research and Development Center who helped conduct this research. Lethbridge Research and Development Center contribution number: 0000.

[2]Corresponding author: tim.mcallister@agr.gc.ca

Received February 5, 2018.
Accepted May 25, 2018.

through the use of novel additives may serve as a means to achieve this objective. Biochar or biocarbon is generated as a result of the partial pyrolysis of OM and although primarily used as a soil amendment (Lehmann and Joseph, 2009), it has recently been reported to enhance feed degradability and possibly lower enteric CH_4 emissions (Leng et al., 2012a,b, 2013).

Biocarbon pyrolyzed at high temperature in a manner that generates a very high surface area is called engineered or activated biocarbon and has been theorized to promote the formation of microbial biofilms in the rumen (Leng et al., 2012a, 2014), a process essential for ruminal feed digestion (McAllister et al. 1994). Further, biochar may lower the production of ruminal CH_4 emissions both *in vitro* (Hansen et al., 2012; Leng et al., 2012a,b) and *in vivo* (Leng et al., 2012c). It has been suggested that biochar reduces ruminal enteric CH_4 emissions by altering rumen microbial biofilms, decreasing rumen methanogens and increasing rumen methanotrophs (Leng et al., 2012a,b,c; Toth and Dou, 2016). However, dose response studies with biocarbon have not been undertaken. The objective of this study was to determine if biocarbon reduces CH_4 production and improves the ruminal fermentation of a high-forage diet in an artificial rumen (RUSITEC).

MATERIALS AND METHODS

Engineered Biocarbon Technology Production

Biocarbon was produced by Cool Planet (CoolFauna, Denver, CO) using a patented proprietary method (Engineered Biocarbon Technology) for manufacturing a consistent, toxin-free product for use in animal diets. The combination of front-end biomass pyrolysis at temperatures between 400 and 600 °C and pyrolysis residence times of only a few minutes produces what is known in the industry as "raw biochar". The raw biochar is then subjected to post-pyrolysis treatment to adjust pH to neutral and remove any remaining polyaromatic hydrocarbons and dioxins, resulting in a hydrophilic product with a consistent particle size. The source of the biocarbon used in this study was from jack pine in the Northern Rocky Mountains of the United States combined with Southern Yellow Pine. Standard pulp woodchips were pyrolyzed and ground following post-treatment processing. Physical and chemical proprieties of biocarbon used in the study are presented in Table 1.

Table 1. Physical properties and chemical composition of biocarbon

Component	Property
Surface area, m^2/g	186
Bulk density kg/m^3	287
Pores volume, cc/g	7.37×10^{-2}
DM, %	96.4
OM, %	97.6
Ash, %	2.4
Carbon, %	76.9
Hydrogen, %	3.0
Nitrogen, %	0.26
Sulfur, %	<0.1
Chlorine, %	0.05
Oxygen, %	17.7
pH	4.8

Experimental Design and Treatments

The experiment was designed as a completely randomized block design with four treatments assigned to sixteen fermentation vessels (n = 4/treatment) in two RUSITEC apparatuses. Treatments were basal diet (no biocarbon supplementation), or biocarbon at 0.5, 1.0, and 2.0% of diet DM. The experiment was 17 d in duration, including a 10-d adaptation, followed by 7 d of sample and data collection. The basal diet consisted of 600 g/kg barley silage, 270 g/kg dry-rolled barley grain, 100 g/kg canola meal, and 30 g/kg supplement (DM basis; Table 2). The supplement contained per kg of dietary DM: 565 g barley grain, 100 g canola meal, 250 g calcium carbonate, 25 g molasses, 30 g salt, 20 g urea, 0.66 g vitamin E 500, and 10 g premix. The premix contained per kg of dietary DM: 15 mg of Cu, 65 of mg Zn, 28 mg of Mn, 0.7 mg of I, 0.2 mg of Co, 0.3 mg of Se, 6,000 IU of vitamin A, 600 IU of vitamin D, and 47 IU of vitamin E. Barley silage was dried at 55 °C for 48 h and all ingredients were ground through a 4-mm screen (Arthur Thomas Co., Philadelphia, PA) prior to generation of the total mixed ration (**TMR**). The diet was weighed (10 g DM) into nylon bags (10 × 20 cm; pore size of 50 µm, Ankom Technology Corp., Macedon, NY) and biocarbon was added to each bag at the desired concentration (0, 0.5, 1.0, and 2.0% of substrate DM) and manually mixed.

Source of Inoculum

Three ruminally fistulated Angus cross cows averaging 768.3 ± 95.1 kg BW were fed a TMR containing 892 g/kg barley silage, 82 g/kg dry-rolled barley grain, and 26 g/kg supplement (DM basis). Cows were handled in accordance with the

Table 2. Ingredient and chemical composition of experimental diets containing increasing levels of biocarbon

	Level of biocarbon (% of diet DM)			
Item	0	0.5	1.0	2.0
Ingredient, g/kg				
Barley silage[1]	600	600	600	600
Barley grain, temper-rolled[2]	270	270	270	270
Canola meal	100	100	100	100
Supplement[3]	30	30	30	30
Biocarbon	—	5	10	20
Chemical composition, % of DM				
DM	90.5	90.4	90.5	90.6
OM	93.7	93.9	93.8	93.8
CP	16.5	16.2	16.1	16.3
ADF	17.3	17.6	18.0	18.4
NDF	36.2	35.8	36.7	36.9
Starch	26.4	26.4	26.1	25.5

[1]91.2% DM, 92.3% OM, 13.2% CP, 25.4% ADF; 45.8% NDF, 18.3% starch.

[2]88.2% DM, 97.1% OM, 15.3% CP, 6.29% ADF; 21.6% NDF, 51.7% starch.

[3]87.9% DM, 95.5% OM, 11.4% CP, 5.38% ADF; 17.4% NDF, 50.6% starch.

guidelines of the Canadian Council on Animal Care (CCAC, 2009), and protocols were reviewed and approved by the Lethbridge Research and Development Centre Animal Care Committee. Solids and liquid contents were collected through a rumen cannula from four locations within the rumen, 2 h after morning feeding. Contents were immediately transported to the laboratory where solid and liquid contents were equally pooled and filtered through four layers of cheesecloth. Inoculum (4 L per cow) was composited, mixed, pH recorded and kept at 39 °C in a water bath prior to introduction into fermenters.

Experimental Apparatus

Two RUSITEC apparatuses (Czerkawski and Breckenridge, 1977; Romero-Pérez et al., 2016, 2017), each equipped with eight 920-mL anaerobic fermenters, were used. Each fermenter was outfitted with an input port for infusion of buffer and an outlet port to collect effluent. Fermenters were housed in a circulating water bath at 39 °C. To initiate fermentation, each fermenter was filled with 200 mL of McDougall's buffer (McDougall, 1948), 700 mL of the strained rumen inoculum, one bag containing 20 g of mixed solid rumen digesta, and one bag containing 10 g of diet. The nylon bag containing solid rumen contents was replaced after 24 h with a bag containing 10 g of diet. Thereafter, one bag was replaced daily at 0900 h, so that each bag remained in each fermenter for 48 h. The artificial saliva was adjusted to a pH of 8.2 and modified to contain 0.3 g/L of $(NH_4)_2SO_4$ and continuously infused into fermenters using a peristaltic pump set to achieve a dilution rate of 2.9%/h. Effluent was collected daily into a 2.0 L Erlenmeyer flask and fermentation gases were collected into reusable 2 L gas-tight collection bags (Curity^R; Conviden Ltd., Mansfield, MA). Anaerobic condition in the fermenters was maintained by flushing the fermenters with CO_2 gas during feed-bag exchange, and temperature was set at 39 °C and controlled by a circulating water bath.

Nutrients Disappearance and Gas Production

Dry matter (DM), OM, CP, ADF, NDF, and starch disappearance from 48-h incubated feed bags was determined from days 11 to 15 of the sampling period. Feed bags were withdrawn from each fermenter and washed under cold tap water until the water was clear. Bags were oven dried at 55 °C for 48 h (AOAC, 1995; method 930.15), and hot-weighed to determine DM disappearance. Residues were pooled over 5 d, ground through a 1-mm screen using a Wiley mill (standard model 4 Arthur Thomas Co.) and analyzed for OM, total N, NDF, ADF, and starch. Ash content was determined by combustion at 550 °C for 5 h, and OM content was calculated as 100 minus the proportion of ash (AOAC, 1995; method 942.05). Total N was determined using a combustion analyzer (NA 2100, Carlo Erba Instruments, Milan, Italy), with CP calculated as N × 6.25. The NDF and ADF contents were determined using the sequential method with an ANKOM200 Fiber Analyzer (Ankom Technology Corp.) using reagents as described by Van Soest et al. (1991), with heat-stable α-amylase (Termamyl 120 L, Novo Nordisk Biochem, Franklinton, NC) and sodium sulfite included in the NDF analysis. Starch was determined by enzymatic hydrolysis of α-linked glucose polymers as described by Rode et al. (1999). Disappearance of OM, CP, NDF, ADF, and starch was determined as the difference between the amount of these components in the substrate before incubation and the amount remaining in the residue after incubation.

Total gas production was recorded daily after 24 h, throughout the experiment using a gas meter (Model DM3A, Alexander-Wright, London, England, UK). A volume of 20 mL gas was sampled from the septum of each collection bag once daily

using a 20-mL syringe and injected into evacuated 6.8-mL exetainers (Labco Ltd., Wycombe, bucks, UK). Methane concentrations were determined using a Varian 4900 gas chromatograph equipped with a GS-CarbonPLOT 30 m × 0.32 mm × 3 µm column and thermal conductivity detector (Agilent Technologies Canada Inc., Mississauga, Ontario, Canada) at an isothermal oven temperature of 35 °C, with helium as the carrier gas (27 cm/s).

Fermentation Characteristics and N Fractions

Fermenter fluid pH (Orion model 260A, Fisher Scientific, Toronto, ON, Canada) and effluent volume from each fermenter were recorded daily at the time of feed-bag exchange. Effluent was sampled for analysis of VFA, NH_3-N, tungstic acid soluble N (**TA-N**), and trichloroaceitic acid soluble N (**TCA-N**). To determine VFA concentrations in fermenter effluent, subsamples (5 mL) were collected directly from the effluent flasks at the time of feed-bag exchange. Samples were placed in screw-cap vials, preserved with 1 mL of 25% (wt/wt) metaphosphoric acid, and frozen at −20 °C until analyzed. At the same time, subsamples (5 mL) of effluent were also collected, placed in screw-cap vials, and preserved with 1 mL of H_2SO_4 (1% vol/vol) for determination of NH_3-N as described by Rhine et al. (1998). Concentration of VFA was quantified using a gas chromatograph (model 5890, Hewlett-Packard Lab, Palo Alto, CA) equipped with a capillary column (30 m × 0.32 mm i.d., 1-µm phase thickness, Zebron ZB-FAAP, Phenomenex, Torrance, CA), and flame ionization detector, with crotonic acid (trans-2-butenoic acid) as an internal standard. The concentrations of VFA and NH_3-N (mmol/L) were multiplied by daily effluent production (L/d) to determine VFA and NH_3-N production (mmol/d). Tungstic acid soluble N and TCA-N were determined as described by Winter et al. (1964). To determine TA-N, subsamples from effluent (4 mL) were added to 1 mL of 10% (wt/vol) sodium tungstate and 1 mL of 1.07 N sulfuric acid. To determine TCA-N, 1 mL of 50% (wt/vol) TCA solution was added to a 4 mL subsample of effluent. After allowing the tubes to stand at 5 °C for 4 h, samples were centrifuged at 9,000 × *g* for 15 min, and the supernatant was analyzed for TA and TCA-N by flash combustion and thermal conductivity detection (Model 1500; Carlo Erba Instruments). Large peptide N (**LPep N**; mg/100 mL) was estimated as (LPep N = TCA-N − TA-N), whereas small peptides (**SPep**) plus AA N (**AA N**) were estimated as (SPep + AA N = TA-N − NH_3-N).

Samples for protozoa counts were collected from days 11 to 15. At the time of feed-bag exchange, 1 mL of liquid was gently squeezed from the 48-h incubated feed bag and preserved (1:1 v/v) in methyl green-formalin-saline solution. The samples were stored in the dark at room temperature until counted by light microscopy using a Levy–Hausser counting chamber (Hausser Scientific, Horsham, PA).

Microbial Protein Synthesis

Bacteria in the fermenters were labeled using ^{15}N as described by Ribeiro et al., 2015. Effluent and feed residue solids were sampled to determine background ^{15}N levels. On day 10, ^{15}N-enriched $(NH_4)_2SO_4$ (Sigma Chemical Co., St. Louis, MO; minimum ^{15}N enrichment 1 g/L) was added to the buffer and infused until the end of the experiment. From day 10, daily effluent accumulation in each flask was preserved with 3 mL of a sodium azide solution to achieve a final concentration of 0.1% wt/vol. On days 16 and 17, the daily total effluent for each fermenter was measured and a subsample (35 mL) was centrifuged (20,000 × *g*, 30 min, 4 °C) for isolation of liquid-associated bacteria (**LAB**). The resulting pellets were washed using deionized water and centrifuged three times (20,000 × *g*, 30 min, 4 °C) prior to suspension in distilled water, freezing, and lyophilizaton for determination of N and ^{15}N.

Feed particle-associated (**FPA**) and feed particle-bound (**FPB**) bacterial fractions were measured from 48-h feed residues on days 16 and 17 by squeezing solids to expel excess liquid from the feed bags. Bags were placed together in a plastic bag with 20 mL of McDougall (1948) buffer and processed for 60 s in a Stomacher 400 Laboratory Blender (Seward Medical Ltd, London, UK). The processed liquid was squeezed out, poured off, and retained. Feed residues were washed twice with 10 mL of McDougall's buffer in each wash. The wash buffer was retained and pooled with the initially expressed fluid to obtain the FPA bacterial fraction, and the total volume was recorded. Washed solid feed residues were considered to represent the FPB bacterial fraction. The FPA bacterial samples collected after stomaching were centrifuged (500 × *g*, 10 min, 4 °C) to remove large feed particles and the supernatant was decanted and centrifuged (20,000 × *g*, 30 min, 4 °C) to isolate a bacterial pellet which was washed three times as described above. The pellet was then resuspended in distilled water and stored at −20 °C.

Washed feed residues (FPB fraction) were dried at 55 °C for 48 h, weighed for DM determination, ball ground (MM400; Retsch Inc., Newtown, PA), and analyzed for total N and ^{15}N by combustion analysis coupled to a mass spectrometer (NA1500, Carlo Erba Instruments). Total daily microbial N production (mg/d) was calculated as the sum of microbial biomass associated with the LAB, FPA, and FPB fractions (Ribeiro et al., 2015).

Statistical Analysis

Data were analyzed as repeated measures according to a completely randomized block design using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). The MIXED model included the fixed effects of treatment, day of sampling, and treatment × day, with the day of sampling as a repeated measure, and the random effect of RUSITEC apparatus. For the repeated measures, various covariance structures were tested with the final structure chosen based on the minimum Akaike's information criteria value. Data were tested for normality of variance. Orthogonal polynomial contrasts were performed to test for linear or quadratic responses to biocarbon, and compare biocarbon to the control. Significance was declared at $P \leq 0.05$ and a trend at $0.05 < P < 0.10$ unless otherwise stated.

RESULTS

Ruminal Fermentation Characteristics

Increasing levels of biocarbon in the diet tended ($P = 0.06$) to linearly increase daily production of NH_3-N (Table 3). Production of total VFA also linearly increased ($P < 0.01$) with increasing levels of biocarbon in the diet. Daily acetate, propionate, branched-chain volatile fatty acids (**BCVFA**) ($P < 0.001$), and butyrate ($P = 0.02$) production also linearly increased (5, 10, or 20 g of biocarbon/kg of substrate DM) with biocarbon, whereas no differences among treatments in the production of valerate, caproate, or the ratio of acetate to propionate were noted. Moreover, biocarbon diets tended to have greater ($P \leq 0.10$) production of daily NH_3-N (7.1 vs. 6.6 mmol/d), acetate (36.5 vs. 34.7 mmol/d), butyrate (7.7 vs. 7.1 mmol/d) and BCVFA (1.86 vs. 1.76 mmol/d), and total VFA ($P = 0.02$; 64.7 vs. 60.0 mmol/d) than the control diet. Methane, expressed as mg/d, g/g of DM incubated, or g/g of DM digested, responded quadratically ($P \leq 0.05$) to increasing levels of biocarbon in the diet. Compared to control, biocarbon diets had lower ($P < 0.02$) CH_4 production (89.6.5 vs. 110.8 mg/d), (8.9 vs. 11.1 g/g of DM incubated), (11.87 vs. 15.2 g/g of DM digested), in all three metrics measured. There were no differences in pH, production of total gas

Table 3. Effect of increasing levels of biocarbon on fermentation and gas production in the RUSITEC

	Level of biocarbon (% of diet DM)					*P* value		
Item	0	0.5	1.0	2.0	SEM	Linear	Quadratic	0 vs. All
pH	6.8	6.9	6.8	6.8	0.04	0.66	0.41	0.49
NH_3-N, mmol/d	6.6	7.0	7.1	7.3	0.40	0.06	0.48	0.06
Total VFA, mmol/d	61.0	61.3	64.2	68.5	1.44	0.005	0.54	0.02
VFA, mmol/d								
Acetate (A)	34.7	34.1	36.4	39.0	1.84	0.001	0.38	0.10
Propionate (P)	14.1	14.6	15.4	16.6	0.45	0.001	0.98	0.008
Butyrate	7.1	7.7	7.3	8.1	0.49	0.02	0.70	0.06
BCVFA[1]	1.76	1.75	1.84	2.0	0.06	0.001	0.29	0.08
Valerate	2.40	2.62	2.48	2.36	0.58	0.58	0.34	0.63
Caproate	0.73	0.69	0.68	0.69	0.70	0.45	0.46	0.26
A:P	2.42	2.35	2.36	2.38	0.08	0.66	0.29	0.24
Gas production, L/d	2.18	1.92	2.15	2.04	0.08	0.63	0.57	0.14
CH_4, % of gas	7.4	6.8	6.8	6.9	0.68	0.02	0.03	0.001
CH_4, mg/d	110.8	82.8	95.3	90.7	9.23	0.07	0.05	0.002
CH_4, g/g DM incubated	11.1	8.3	9.5	8.9	0.92	0.04	0.05	0.001
CH_4, g/g DM digested	15.2	11.0	12.8	11.8	1.22	0.06	0.03	0.001
Protozoa, $\times 10^4$/mL	5.2	4.3	6.1	5.5	0.41	0.21	0.68	0.91

A:P = acetate to propionate ratio.

[1]Branched-chain volatile fatty acids (isobutyrate + isovalerate).

or numbers of total protozoa between control and biocarbon diets.

Nutrient Disappearance and Bacterial Protein Synthesis

Increasing the level of biocarbon in the diet resulted in a linear increase in degradability of DM (P = 0.006), CP (P = 0.02), ADF and NDF (P = 0.0001), and a quadratic response for OM (P = 0.04) degradability, with no effect on starch degradability (Table 4). Moreover, biocarbon diets versus control had greater (P < 0.001) degradability of DM (74.9 vs. 73.2%), CP (86.2.3 vs. 843%), NDF (44.8 vs. 39.9%), and ADF (36.75 vs. 29.9%). Effluent and total (LAB + FPB + FPA) microbial N production was linearly increased (P ≤ 0.01) with increasing biocarbon in the diet. Compared to control, biocarbon treatments had greater (P = 0.02) total microbial N production (96.1 vs. 85.7 mg/d) and microbial N production in the LAB fraction (68.3 vs. 56.9 mg/d), whereas microbial N production in FPB fraction was reduced (P = 0.04) with biocarbon diets versus control (21.3 vs. 23.3 mg/d). There were no differences (P > 0.05) in microbial N production in FPA fraction and concentrations of large and SPep among treatments.

DISCUSSION

Biochar or biocarbon, is typically a byproduct of the forestry industry obtained by pyrolysis of wood biomass and may have potential as a feed additive in ruminant diets due to possible enhancement of microbial fermentation (McFarlane et al., 2017). It has been suggested that biocarbon also has the potential capacity to alter ruminal microbiota and decrease enteric CH_4 emissions. Very few studies have investigated the impact of increasing concentrations of biocarbon in the diet on ruminal nutrient digestibility, VFA production, and CH_4 production.

Ruminal Fermentation Characteristics

Increased VFA production is consistent with the observed improvement in DM, OM, CP, and fiber degradability with increasing levels of biocarbon in the diet. The greater concentration of acetate with biocarbon is also consistent with an increase in fiber digestion. In agreement with this study, biochar increased total VFA production from silage and hay in vitro (Pereira et al., 2014). Moreover, in an early *in vivo* study using goats, Garillo et al. (1994) reported that addition of 0.6% biocarbon

Table 4. Effect of increasing levels of biocarbon on nutrient disappearances and microbial N synthesis in the RUSITEC

	Level of biocarbon (% of diet DM)					*P* value		
Item	0	0.5	1.0	2.0	SEM	Linear	Quadratic	0 vs. All
Nutrient disappearance,[1] %								
DM	73.2	74.8	74.5	75.5	0.88	0.006	0.38	0.006
OM	72.9	75.0	74.6	75.2	2.23	0.005	0.04	0.001
CP	84.3	85.8	86.4	86.5	1.28	0.02	0.10	0.007
NDF	39.9	43.8	44.3	46.4	1.63	0.001	0.09	0.001
ADF	29.9	35.3	35. 5	38.2	1.57	0.001	0.08	0.001
Starch	98.0	98.2	98.0	98.1	0.57	0.83	0.87	0.66
Production of microbial N,[2] mg/d								
Total	85.7	92.1	95. 8	100.1	8.27	0.01	0.43	0.02
LAB[3]	56.9	64.6	68.0	72.3	6.61	0.02	0.39	0.02
FPB[4]	23.3	20. 8	21.4	21.1	2.41	0.19	0.20	0.04
FPA[5]	5.7	6.4	6.5	6.9	0.58	0.16	0.71	0.20
N fractions,[1] mg/100 mL								
LPep N[6]	19.0	16.1	18.9	15.6	1.64	0.27	0.84	0.30
SPep + AA N[7]	6.2	7.9	7.6	7.6	1.01	0.49	0.43	0.25

[1]Samples from days 11 to 15.
[2]Samples from days 17 to 18.
[3]Liquid associate bacteria.
[4]Feed particle-bound bacteria.
[5]Feed particle-associated bacteria.
[6]Large peptides.
[7]Small peptides plus AA.

to the diet increased ruminal total VFA and propionate concentrations. However, McFarlane et al. (2017) examined three different biomass sources of biochar, chestnut oak, yellow popular, and white pine, processed to two different particle sizes [fine (<178 µm) and coarse (>178 µm)] at 81 g/kg DM in an orchard grass-based diet *in vitro* and observed that acetate, propionate, and butyrate production were not affected by biomass source or particle size. Differences in responses of biocarbon on VFA production may be attributed to the differing biomass sources, processing techniques, or dosages. The biomass source used to produce biocarbon could lead to differential responses in rumen fermentation due to variation in the structure and composition of the source material (McFarlane et al., 2017). Secondary processing of biochar to biocarbon and the associated increase in particulate surface area (McFarlane et al., 2017) could also influence the biological responses of the ruminal microbial community to these additives.

Consistent with our results, previous studies have shown that addition of biocarbon did not affect total *in vitro* gas production (Hansen et al., 2012; Leng et al., 2012a; Pereira et al., 2014). Moreover, we observed that biocarbon reduced overall CH_4 production, which is consistent with previous studies where it reduced CH_4 emissions arising from ruminal microbial fermentation both *in vitro* (Hansen et al., 2012; Leng et al., 2012a,b; Phanthavong et al., 2015; Phonethep et al., 2017; Sengsouly and Preston, 2016a) and *in vivo* (Leng et al., 2012c; Sengsouly and Preston, 2016b). Hansen et al. (2012) tested four types of biochar (gasified, wood-based, straw based, and activated charcoal) at 9% of dietary DM and found that in ruminal batch cultures it reduced CH_4 production by 11% to 17% as compared to controls. A study with rice hull–based biochar showed that levels of 0, 1, 2, 3, 4, 5% of dietary DM in a mixed diet with cassava root meal and urea decreased CH_4 (mL/24 h) production by 11% to 13% at 1% of dietary DM, with no further reduction at higher concentrations (Leng et al., 2012a). Biochar also decreased ruminal CH_4 emissions *in vitro* when included at 1% of DM in dried, ensiled, or fresh cassava root diets supplemented with urea and cassava meal as sources of N (Vongkhamchanh et al., 2015). Leng et al. (2012c) fed cattle a basal diet of fresh cassava root chips, with biochar at 0.6% of dietary DM over 98 d and observed that CH_4 production was decreased by 22%. Further evidence that biochar affects rumen microbial activity was shown in an *in vitro* study in which CH_4 production was lower in rumen fluid obtained from cattle previously fed biochar as compared to cattle that did not receive this additive (Leng et al., 2012c).

It has been suggested that biochar reduces ruminal CH_4 production by altering rumen microbial biofilms, decreasing rumen methanogens and increasing rumen methanotrophs (Leng et al., 2012a,b,c; Toth and Dou, 2016). Although there is little direct evidence that has supported these responses in the rumen, the ability of biochar to reduce CH_4 emission in rice paddy soils has been linked to an increase in the abundance of methanotrophs relative to methanogens (Feng et al., 2012). Other explanations for the biocarbon-mediated reduction in CH_4 emissions in the rumen have included its high specific surface area and porous structure (Toth and Dou, 2016) possibly, adsorbing the CH_4 produced (Hansen et al., 2012). However, given the large volumes of CH_4 produced in the rumen relative to the low levels of biocarbon included in the diet, it is difficult to fathom how this relationship could cause a meaningful decline in enteric CH_4 emissions. The possibility of the direct sequestration of CH_4 by biocarbon in the rumen requires further investigation.

In the RUSITEC, numbers of protozoa did not differ as result of increasing levels of biocarbon in the diet. This is in agreement with a previous study where addition of 0.3% and 0.6% biocarbon to the diet did not alter the number of protozoa in the rumen of goats (Garillo et al., 1994). Others have suggested that biocarbon at 1% of dietary DM is the optimal level of inclusion to most effectively lower enteric CH_4 emissions (Leng et al., 2012a,b,c; Vongkhamchanh et al., 2015). Phanthavong et al. (2015) also reported a 7% reduction of CH_4 within 24 h in an *in vitro* study using cassava in rumen fluid with 1% biochar. It will be important to assess the CH_4-reducing capacity of biocarbon arising from different biomass sources over a range of dosages to fully define the potential of this additive to lower enteric CH_4 emissions. In the current study, the lowest CH_4 production was achieved at level of 50 g biocarbon/kg substrate DM, but higher inclusion of biocarbon promoted nutrient digestibility, VFA, and microbial N production.

Nutrient Disappearance and Bacterial Protein Synthesis

The increase in DM, OM, CP, and fiber disappearance suggests that biocarbon may be promoting the development of a ruminal microbial population that is more efficient at degrading feed.

Changes in fermentation are not unexpected as antimicrobial properties within biochar may specifically impact some members of the rumen microbiota (Van et al., 2006; Thu et al., 2010; Al-Kindi, 2015). Leng et al. (2012a) found that DM degradability *in vitro* was increased when biochar from rice husks was added to a cassava-based diet with either potassium nitrate or urea as a N source. Erickson et al. (2011) fed lactating Holstein cows a diet containing 60% poor-quality or good-quality corn silage, top-dressed with biocarbon at level of 0, 20, or 40 g/cow. Cows fed biocarbon had higher NDF, hemicellulose, and CP digestibility compared to controls. However, these improvements were not observed in cows fed good-quality corn silage. Furthermore, Van et al. (2006) fed growing goats a grass and concentrate mixture supplemented with biocarbon produced from bamboo at levels of 0, 0.5, 1.0, or 1.5% of BW and found that apparent digestibility of DM, OM and CP was improved ($P < 0.05$) compared to the control. Contrarily, Hansen et al. (2012) included biochar produced from straw or wood at 9% of dietary DM in a ruminal *in vitro* study and found that DM and cell wall degradability were not affected. However, the biocarbon used in our study and by Leng et al. (2012ac) may have created conditions that were more emendable to improving the digestive efficiencies of rumen microbes compared to the biochar sources used by Hansen et al. (2012). Differences in responses of biocarbon among studies may be due to differing sources of biomass, particle size, and pyrolysis conditions, which may alter rumen fermentation to varying degrees (Hansen et al., 2012; McFarlane et al., 2017). The biochar-starting material as well as temperature of pyrolysis may also contribute to the absorptive potential of biochar (Lehmann and Joseph, 2009). When biochar is subjected to low temperatures of pyrolysis during carbonization, it has been noted that surface area per unit weight is reduced, potentially decreasing its capacity to adsorb nutrients and provide a habitat suitable for the formation of biofilms (Leng, 2014). Potential impacts on microbial–forage interactions and forage digestibility could be dependent on the source of biomass, particle size of biochar, and other factors that affect the overall structure and porosity of the material (McFarlane et al., 2017). Leng et al. (2012a) reported that the percentage of DM solubility was increased when biochar was produced from rice husks exposed to high pyrolysis temperatures (~900 °C).

There was no effect of biocarbon on ruminal concentrations of large or SPep; even though ruminal protein degradation and NH_3 concentrations were increased as a result of the inclusion of biocarbon in the diet. Results suggest that biocarbon did not target any specific proteolytic steps in the rumen. The observed increase in NH_3-N and BCVFA suggests that biocarbon increased AA deamination. There is lack of information on the effects of biocarbon on in vitro microbial N synthesis. In the current study, the increase in total bacterial protein synthesis was mainly due to increased OM, NDF, and ADF digestibility as a result of addition of biocarbon to the diet. Van et al. (2006) reported that feeding 1.5 g of biocarbon generated from bamboo per kg live BW of goats improved CP digestibility and N retention. Moreover, Al-Kindi (2015) reported that adding biocarbon at 1.5% and 3% of diet DM to 50% grass hay and 50% concentrate pelleted diet improved the efficiency of rumen microbial protein synthesis in goats.

CONCLUSIONS

Addition of biocarbon to a high-forage diet up to 2% of diet DM improved *in vitro* ruminal fermentation, nutrient disappearance, and microbial protein synthesis, and reduced enteric CH_4 production. The lowest CH_4 production was achieved with 0.5% of biocarbon of dietary DM. Comparison of fermentation responses to biocarbon can be challenging due to differences in biomass sources and pyrolysis conditions. Further *in vivo* studies on the mechanistic factors responsible for lowered CH_4 production and improved nutrient digestibility are required to more accurately predict ruminal responses to inclusion of biocarbon in ruminant diets.

LITERATURE CITED

Al-Kindi, A. 2015. Influence of quebracho tannin extract and activated charcoal on nutrient intake and digestibility, digesta passage, nitrogen balance, and quality of faecal excreta in goats. PhD Diss. Kassel Univ., Witzenhausen, Germany.

AOAC.1995. Official methods of analysis. 16th ed. Assoc. Off. Anal. Chem., Arlington, VA.

Canadian Council on Animal Care. 2009. Guide to the care and use of farm animals in research, teaching and testing. CCAC, Ottawa, ON.

Czerkawski, J. W., and G. Breckenridge. 1977. Design and development of a long-term rumen simulation technique (Rusitec). Br. J. Nutr. 38:371–384.

Erickson P. S., N. L. Whitehouse, and M. L.Dunn. 2011. Activated carbon supplementation of dairy cow diets: effects on apparent total-tract nutrient digestibility and taste preference. Prof. Anim. Sci. 27: 428–434. doi:10.15232/S1080-7446(15)30515-5

Feng Y., Y. Xu, Y. Yu, Z. Xie, and X. Lin. 2012. Mechanisms of biochar decreasing methane emission from Chinese paddy soils. Soil Biol. Biochem. 46:80–88. doi:10.1016/j.soilbio.2011.11.016

Garillo, E. P., R. Pradhan, and H. Tobioke. 1994. Effects of activated charcoal on ruminal characteristics and blood profiles in mature goats. West Japan J. Anim. Sci. 37: 85–89. doi:10.11461/jwaras1968.37.85

Hansen, H. H., I. M. L. D. Storm, and A. M. Sell. 2012. Effect of biochar on in vitro rumen methane production. Acta Agric. Scand. A: Anim. Sci. 62:305–309. doi:10.1080/09064702.2013.789548

Kammann C., J. Ippolito, N. Hagemann, N. Borchard, M. Cayuela, J. Estavillo, T. Fuertes-Mendizabal, S. Jeffery, J. Kern, J. Novak, et al. 2017. Biochar as a tool to reduce the agricultural greenhouse-gas burden - knowns, unknowns and future research needs. J. Environ. Eng. Landsc. 25:114–139. doi:10.3846/16486897.2017.1319375

Lehmann, J., and S. Joseph. 2009. Biochar for environmental management: an introduction. In: J. Lehmann, and S. Joseph, editors, Biochar for environmental management, science and technology. Earthscan, London. p. 1–12.

Leng, R. A. 2014. Interactions between microbial consortia in biofilms: a paradigm shift in rumen microbial ecology and enteric methane mitigation. Anim. Prod. Sci. 54:519–543. doi:10.1071/AN13381

Leng, R. A., S. Inthapanya, and T. R. Preston. 2012a. Biochar lowers net methane production from rumen fluid in vitro. Livestock Res. Rural Develop. 24:103. http://www.lrrd.org/lrrd24/6/sang24103.htm (accessed 4 May 2015).

Leng, R. A., S. Inthapanya, and T. R. Preston. 2012b. Methane production is reduced in an in vitro incubation when the rumen fluid is taken from cattle that previously received biochar in their diet. Livestock Res. Rural Develop. 24:211. http://www.lrrd.org/lrrd24/11/ sang24211.htm (accessed 4 May 2015).

Leng, R. A., S. Inthapanya, and T. R. Preston. 2013. All biochars are not equal in lowering methane production in in vitro rumen incubations. Livestock Res. Rural Develop. 25:106. http://www.lrrd.org/lrrd25/6/leng25106.htm.

Leng, R. A., T. R. Preston, and S. Inthapanya 2012c. Biochar reduces enteric methane and improves growth and feed conversion in local "Yellow" cattle fed cassava root chips and fresh cassava foliage. Livestock Res. Rural Develop. 24:199. http://www.lrrd.org/lrrd24/11/ leng24199.htm.

McAllister, T. A., H. D. Bae, G. A. Jones, and K. J. Cheng. 1994. Microbial attachment and feed digestion in the rumen. J. Anim. Sci. 72:3004–3018.

McDougall, E. I. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochem. J. 43: 99–109. doi:10.1042/bj0430099

McFarlane, Z.D., P. R. Myer, E. R. Cope, N. D. Evans, T. C. Bone, B. E. Biss, and J. T. Mulliniks. 2017. Effect of biochar type and size on in vitro rumen fermentation of orchard grass hay. Agric. Sci. 8:316–325. doi:10.4236/as.2017.84023

Myhre, G., D. Shindell, F.-M. Bréon, W. Collins, J. Fuglestvedt, J. Huang, D. Koch, J.-F. Lamarque, D. Lee, B. Mendoza, et al. 2013. Anthropogenic and natural radiative forcing. In: T. F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P. M. Midgley, editors, Climate change 2013: the physical science basis. contribution of working group i to the fifth assessment report of the intergovernmental panel on climate change. Cambridge University Press, Cambridge, United Kingdom. p. 659–740. doi:10.1017/CBO9781107415324.018

Pereira, C., R. Muetzel, S. Camps, M. Arbestain, P. Bishop, K. Hina, and M. Hedley. 2014. Assessment of the influence of biochar on rumen and silage fermentation: a laboratory-scale experiment. Anim. Feed Sci. Tech. 196:220–231. doi:10.1016/j.anifeedsci.2014.06.019

Phanthavong V., N. Viengsakoun, I. Sangkhom, and T. R. Preston. 2015. Effect of biochar and leaves from sweet or bitter cassava on gas and methane production in an in vitro rumen incubation using cassava root pulp as source of energy. Livestock Res. Rural Develop. 27, Article #72. http://www.lrrd.org/lrrd27/4/phan27072.html (accessed 4 April 2017).

Phonethep, P., P. Sengsouly, T. R. Preston and R. A. Leng. 2017. Methane production in an in vitro rumen fermentation of molasses-urea was reduced by supplementation with fresh rather than dried cassava (*Manihot esculenta*, Crantz) leaves and by biochar. Livestock Res. Rural Develop. 29, Article #41. http://www.lrrd.org/lrrd29/3/ppho29041.html (accessed 6 November 2017).

Rhine, E. D., G. K. Sims, R. L. Mulvaney, and E. J. Pratt. 1998. Improving the Berthelot reaction for determining ammonium in soil extracts and water. Soil Sci. Soc. Am. J. 62:473–480. doi:10.2136/sssaj1998.03615995006200020026x

Ribeiro, G. O., L. C. Gonçalves, L. G. Pereira, A. V. Chaves, Y. Wang, K. A. Beauchemin, and T. A. McAllister. 2015. Effect of fibrolytic enzymes added to a *Andropogon gayanus* grass silage-concentrate diet on rumen fermentation in batch cultures and the artificial rumen (Rusitec). Animal 9:1153–1162. doi:10.1017/S1751731115000221

Rode, L. M., W. Z. Yang, and K. A. Beauchemin. 1999. Fibrolytic enzyme supplements for dairy cows in early lactation. J. Dairy Sci. 82:2121–2126. doi:10.3168/jds.S0022-0302(99)75455-X

Romero-Pérez, A. E. K., Okine, L. L. Guan, S. M. Duval, M. Kindermann, and K. A. Beauchemin. 2016. Effects of 3-nitrooxypropanol and monensin on methane production using a forage-based diet in Rusitec fermenters. Anim. Feed Sci. Technol. 220: 67–72. doi:10.1016/j.anifeedsci.2016.07.013

Romero-Pérez, A. E. K., Okine, L. L. Guan, S. M. Duval, M. Kindermann, and K. A. Beauchemin. 2017. Rapid Communication: evaluation of methane inhibitor 3-nitrooxypropanol and monensin in a high-grain diet using the rumen simulation technique (Rusitec). J. Anim. Sci. 95:4072–4077. doi:10.2527/jas2017.1896

Sengsouly P., and T. R. Preston. 2016a. Effect of rice-wine distillers' byproduct and biochar on growth performance and methane emissions in local "Yellow" cattle fed ensiled cassava root, urea, cassava foliage and rice straw. Livestock Res. Rural Develop. 28, Article #178. http://www.lrrd.org/lrrd28/10/seng28178.html (accessed 5 June 2017).

Sengsouly P., and T. R. Preston. 2016b. Effect of rice-wine distillers' byproduct, biochar and sweet or bitter cassava leaves on gas production in an in vitro incubation using ensiled cassava root as substrate. Livestock Res. Rural Develop. 28, Article #190. http://www.lrrd.org/lrrd28/10/seng28190.html (accessed 5 June 2017).

Thu, M., S. Koshio, M. Ishikawa, and S. Yokoyama. 2010. Effects of supplementation of dietary bamboo charcoal on growth performance and body composition of juvenile Japanese

flounder, *Paralichthys olivaceus*. J. World Aquacult. Soc. 41: 255–262. doi:10.1111/j.1749-7345.2010.00365.x

Toth J. D., and Z. Dou, 2016. Use and impact of biochar and charcoal in animal production systems. In: M. Guo, Z. He, and M. Uchimiya, editors, Agricultural and environmental applications of biochar: advances and barriers. Soil Science Society of America, Inc., Madison, p. 199–224. doi:10.2136/sssaspecpub63.2014.0043.5

US-EPA. 2011. Inventory of U.S. greenhouse gas emissions and sinks: 1990 – 2009. United States Environmental Protection Agency, Washington, DC, EPA, July 2011.

Van D. T. T., N. T. Mui, and I. Ledin. 2006. Effect of method of processing foliage of *Acacia mangium* and inclusion of bamboo charcoal in the diet on performance of growing goats. Anim. Feed Sci. Tech. 130:242–256. doi:10.1016/j.anifeedsci.2006.01.008

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583–3597. doi:10.3168/jds.S0022-0302(91)78551-2

Vongkhamchanh, B., S. Inthapanya, and T. R. Preston. 2015. Methane production in an in vitro rumen fermentation is reduced when the carbohydrate substrate is fresh rather than ensiled or dried cassava root, and when biochar is added to the substrate. Livestock Res. Rural Develop. 27, Article #208. http://www.lrrd.org/lrrd27/10/bobb27208.html (accessed 4 February 2017).

Winter, K. A., R. R. Johnson, and B. A. Dehority. 1964. Metabolism of urea nitrogen by mixed cultures of rumen bacteria grown on cellulose. J. Dairy Sci. 47:793–797. doi:10.3168/jds.S0022-0302(64)88766-X