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Effects of biochar source, level of inclusion and particle size on *in vitro* dry matter disappearance, total gas and methane production and ruminal fermentation parameters in a barley silage-based diet

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Abstract

Tamayao, P. J., Ribeiro, G. O., McAllister, T. A., Ominski, K. H., Okine, E. K., McGeough, E. J. This study evaluated the effects of biochar differing in source, inclusion level, and particle size on DM disappearance (DMD), total gas and methane (CH₄) production, and ruminal fermentation in a barley silage-based diet. The seven biochar products used were coconut (CP001 and CP014) or pine (CP002, CP015, CP016, CP023, CP024)-based. Experiment 1 evaluated these biochars at 4.5, 13.5 and 22.5% level of diet inclusion, whereas Experiment 2 evaluated CP002, CP016 and CP023 at 2.25 and 4.50% of the diet at < 0.5, 0.5-2.0, > 2.0 mm particle size. Data were analyzed using PROC MIXED in SAS as a randomized complete block design, with biochar source, inclusion level and particle size (Exp. 2 only) as fixed effects with run and replicate as random effects. Increasing level of biochar inclusion linearly ($P < 0.01$) decreased DMD in Exp. 1 and did not influence DMD ($P > 0.05$) in Exp. 2. Total gas, CH₄ (mL/g DMD) and ruminal fermentation parameters were not affected by product, inclusion level or particle size ($P > 0.05$). In conclusion, biochar of varying source and particle size did not mitigate CH₄ production, but reduced DMD at higher inclusion levels in the barley silage-based diet.

Keywords: biochar, methane, ruminal fermentation, batch culture, total mixed ration

Abbreviations

DM, dry matter; **DMD**, DM disappearance; **VFA**, volatile fatty acids; **BCVFA**, branched VFA

Introduction

Methane (CH_4) is a natural by-product of digestion in ruminants as feed material is broken down by a plethora of rumen microbes via microbial fermentation (McAllister et al. 1994; Johnson and Johnson 1995). Thus, rumen-centric strategies (such as dietary manipulation) have been examined with the objective of mitigating CH_4 production while improving animal productivity (Martinez-Fernandez et al. 2016; Haque et al. 2018). Hence, there has been a significant growing interest in the inclusion of novel feed additives in ruminant diets (Terry et al. 2018).

Biochar is a carbon(C)-rich by-product produced from the manufacturing of activated carbon and is used in industrial and soil purification processes (Horne and Williams 1996). Biomass sources of biochar include manures, agricultural crop residues and lignocellulose-rich sources such as wood and straws. It has been suggested that biochar can mitigate soil CH_4 emissions by increasing the ratio of methanotrophs to methanogens (with potential inhibitory effects on methanogens) leading to increased CH_4 oxidation and lowered CH_4 emissions (Feng et al. 2012; Leng 2014). It has been proposed that in manure and soil, the structure and porosity of biochar mediate electron transfer among bacterial species (Chen et al. 2008; Kammann et al. 2017). Biochar and activated charcoal have been used as feed additives to ruminant diets (Man et al. 2020) and it is speculated that in the rumen, it may act as a digestive catalyst by promoting biotic to abiotic assemblages that enhance microbial fermentation (Lehmann and Joseph 2014; Leng 2014). This is achieved as biochar can be a solid medium mediating electron transfers between microbes and terminal acceptors (Watarai et al. 2008; Leng et al. 2012a, b, c; Mitsumori et al. 2012). Moreover, due to its electron-mediating capabilities within microbial communities, biochar may lower enteric CH_4 by shifting ruminal fermentation towards propionate production, which acts as an alternate H_2 sink to the reduction of CO_2 to CH_4 by methanogens (Mitsumori et al. 2012; Leng

et al. 2014; Saleem et al. 2018). The electron-mediating capabilities of biochar, in addition to its physical (pore size, pore volume, surface area) and chemical (pH, C content) characteristics, are influenced by the originating biomass, as well as pyrolysis conditions, temperature, post-pyrolysis treatment (if applied) and processing (Lehmann and Joseph 2014; Schmidt et al. 2019).

Several *in vitro* studies have evaluated the effects of biochar on CH₄ and associated parameters, with a range observed in its mitigation potential from no effect (McFarlane et al. 2017; Terry et al. 2019; Teoh et al. 2019; Tamayao et al. 2021) to a 25% reduction (Saleem et al. 2018). There is also a lack of knowledge in the assessment of biochar differing in particle size, and how these differences might impact feed digestibility, ruminal fermentation and gas and CH₄ production. Smaller biochar particle size (< 0.178 mm) is reported to increase gas production (McFarlane et al. 2017) and improve forage digestibility *in vitro* (Saleem et al. 2018); however, this is not consistent in the published literature. Hence, the evaluation of biochar effects using *in vitro* approaches can be used to determine the intrinsic properties of biochar that result in favorable outcomes in terms of CH₄ emissions. The experiments in this study assessed the effects of seven biochar products differing in source, inclusion level and particle size on *in vitro* DMD, total gas and CH₄ production and ruminal fermentation parameters of a barley silage-based diet typical of that offered to growing cattle in western Canada.

Materials and methods

Animal care and handling

The animals used in the study were handled in accordance to the Canadian Council on Animal Care Guidelines (AC10997; CCAC 2009), with experimental procedures approved by the University of Manitoba Animal Care Committee.

Basal experimental diet, treatments and experimental design

The two experiments were conducted as randomized complete block designs. Each block was a complete experimental run conducted at different times (three runs per experiment). Within each run, there were three vials per treatment (the vials were randomly assigned on trays, i.e., levels within the incubator, ensuring that there was one vial from each treatment on each level to account for any gradient effect the incubator might have). Each level of the incubator was considered a replication. The basal experimental diet utilized in the *in vitro* incubation was a TMR composed of 600 barley silage, 270 dry-rolled barley grain, 100 canola meal and 30 mineral/vitamin supplement (g kg^{-1} DM basis). The supplement consisted of 565 barley grain, 250 calcium carbonate, 100 canola meal, 30 salt, 25 molasses, 10 mineral/vitamin premix and 0.66 vitamin E (g kg^{-1} DM basis).

The biochar products used in this study were supplied by Cool Planet® (Greenwood Village, CO, USA) and pyrolyzed from coconut husks (CP001, CP014) or pine wood chips (CP002, CP015, CP016, CP023, CP024). In-depth analyses (ASTM method 1762 for proximate analyses) of the C content, pH and particle size distribution of all biochars were provided by Innotech Alberta® (Edmonton, AB, CA) and are reported in Table 1. The basal experimental diet

(0.5 g DM) without biochar was included as a control in both experiments, whereas biochar treatments consisted of 0.5 g of TMR plus biochar product at the specified inclusion level (Exp. 1 and 2) and particle size (Exp. 2).

In Exp. 1, the seven biochar products listed above were assessed at three inclusion levels which were 0.5, 1.5 and 2.5 mg mL⁻¹ inoculum (4.5, 13.5 and 22.5% diet DM) over a 24-h incubation period. In Exp. 2, CP002, CP016 and CP023 were selected to provide a range of physical (bulk density, surface area, pore volume, particle size distribution) and pH across the available products. These products were assessed at two inclusion levels which were 0.25 and 0.5 mg mL⁻¹ (2.25 and 4.50% diet DM) and three particle sizes (< 0.5, 0.5-2.0, > 2.0 mm) over a 48-h incubation period. To achieve the desired particle size, the biochar products were ground using a Wiley Mill (Thomas Scientific., Swedesboro, NJ, USA) with the corresponding sieve size of 0.1, 0.5 and 2.0 mm.

Inoculum source and in vitro incubation

Three ruminally-cannulated Aberdeen Angus heifers were used as rumen fluid donors for both experiments and were housed at the University of Manitoba Glenlea Research Station. Heifers were offered the same TMR as utilized in the *in vitro* incubation (described above) and fed once daily (0800-0830h) for 14 d prior to rumen sampling in both experiments. Both *in vitro* experiments were conducted in accordance to the procedures described by Menke et al. (1979). The diet and biochar products were oven-dried at 55°C for 48 h and thereafter, the basal diet was ground (Thomas Scientific., Swedesboro, NJ, USA) through a 1 mm screen. In Exp. 1, all biochar products were ground to 1 mm and in Exp. 2, to the specified particle sizes. The diet was weighed (0.5 g DM) individually into incubation vials (capacity 120 mL) and the respective type

and amount of biochar added for each incubation. Buffer mineral solution (McDougall 1948) was prepared and maintained at 39°C, and continually flushed with CO₂ prior to and during dispensing (total of 3 h). Rumen fluid was collected 2 h post-feeding from donor heifers from four different locations in the rumen and strained through Pecap mesh (mesh size 250 µm; PA66CG-250 136 cm, Sefar Nyltal, Gilbert Saguenay, QC, CA). The fluid was then composited in pre-warmed insulated containers and filtered through three layers of cheese cloth in the laboratory. Rumen fluid was transferred into a pre-heated 4 L glass jar maintained at 39°C in a water bath where CO₂ was continuously flushed to maintain anaerobic conditions. The inoculum, consisting of 15 mL rumen fluid and 30 mL buffer mineral solution, was dispensed into each vial and sealed with butyl rubber stoppers and aluminum crimp caps. Treatment and blank (inoculum only) vials were then placed on orbital shakers (speed at 60 rpm, TYZD-III orbital shaker; Jiangsu Tenlin Instrument, Jiangyan, China) inside an incubator set at 39 °C (VWR Scientific, Model 2020, Mississauga, ON, CA) for 24 h and 48 h in Exp. 1 and 2, respectively. Treatments were blank-corrected for measurements in both experiments.

Experimental Measurements

Dry matter disappearance

The DMD (expressed as %) was determined as the difference between the substrate DM (TMR with or without biochar) and dry weights of sample residues in the diet before and after incubation, divided by the substrate DM in the diet prior to incubation. Solid residues from each vial were transferred into pre-weighed falcon tubes (50 mL) and centrifuged at $1575 \times g$ (Thermo Scientific, Sorvall Legend X1R, NH, USA) for 15 min at 4 °C. The resulting supernatant was discarded, after filtering through mesh (mesh size 250 µm; PA66CG-250 136 cm, Sefar Nyltal,

Gilbert Saguenay, QC, CA) and the residue was retained. Incubation vials were flushed with distilled water and centrifuged a total of three times to ensure that all solid contents were collected. After centrifugation, the final supernatant was filtered and discarded and the solid pellets were oven-dried for 48 h at 55 °C. These samples were weighed and the residue weight recorded to calculate DMD.

Total gas and CH₄ production

For Exp. 1, gas was collected at 3, 6, 9, 12 and 24 h, whereas for Exp. 2 gas additional samples were collected at 18, 36 and 48 h. Gas pressure was recorded at all time-points using a pressure transducer (Traceable® pressure calibrator, model 33500-086, VWR international, Friendswood, TX, USA). A 10 mL gas sample was collected at each timepoint from each vial via syringe (20 mL) and injected (25-gauge, ½ needle) into 6.8 mL exetainers (Labco, Ltd., Wycombe, London, UK). The gas pressure measurements obtained at each time point were summed to calculate total gas production using equation 3 in the analysis described by Romero-Perez and Beauchemin (2018). Methane production was determined from gas samples via gas chromatography (GC; Agilent 7890B series GC custom, Agilent Technologies Canada Inc., Mississauga, ON, CA). The GC apparatus had an inlet temperature of 150 °C and a constant pressure of 30 psi, with a total septum purge flow of 3 mL/min. Gas samples were manually injected into the front inlet detector (250 °C; airflow rate of 450 mL min⁻¹; H₂ flow rate 70 mL min⁻¹). In both experiments, total gas and CH₄ production were expressed as cumulative values from all time points and were expressed as mL/g DMD and mL/g DM incubated.

Ruminal fermentation parameters

Following incubation in each experiment, vials were immediately placed on ice and the pH of the liquid fractions recorded. For VFA analysis, 3 mL of the fermented fluid was collected from each vial into pre-filled tubes containing 25% metaphosphoric acid (0.6 mL) as per the techniques of Erwin et. al (1961) and stored at -20 °C until analyzed. For the analysis, VFA samples were thawed and mixed with 25% sodium hydroxide (0.240 mL) and 0.3 M oxalic acid (0.384 mL) and centrifuged at $1008 \times g$ for 20 min (Thermo Scientific, Sorvall Legend X1R, NH, USA). The resulting supernatant (1 mL) was analyzed using GC (Varian 3900, Walnut Creek, CA, USA) equipped with an auto sampler (CP 8400, Walnut Creek, CA, USA). Total VFA concentrations as well as concentrations of acetate, propionate, butyrate, branched VFAs (BCVFA; isovalerate and isobutyrate) and the acetate to propionate (A: P) ratio were determined (Khalouei et al. 2020).

For $\text{NH}_3\text{-N}$ analysis, 3 mL of fluid collected from each vial was mixed with 7.2 N sulfuric acid (0.6 mL) and the samples stored at -20 °C. Subsequently, samples were thawed and prepared via the Indole-Phenol method (Novosamsky et al. 1974) and analyzed using a UV spectrophotometer (Ultrospec 3100 pro UV/Visible, Cambridge, England, UK). The $\text{NH}_3\text{-N}$ concentrations were determined via measuring absorbance at 655 nm (Microplate Manager 4.0, Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Data were analyzed using PROC MIXED in SAS (SAS, 2018; university edition for Linux, SAS Institute, Inc., Cary, NC, USA). Both experiments were conducted as a randomized complete block design, with three experimental runs (as blocks) and each treatment having three laboratory replicates. In Exp. 1, the model included the fixed effects of biochar product, biochar inclusion

level and biochar product $\times$ biochar inclusion level interaction. In addition, run, replicate within run as well as biochar product $\times$ biochar inclusion level $\times$ run were considered random effects. In Exp. 2, the fixed effects were biochar product, biochar inclusion level, particle size and biochar product $\times$ biochar inclusion level; biochar product $\times$ biochar particle size; biochar particle size $\times$ biochar inclusion level; biochar product $\times$ biochar inclusion level $\times$ biochar particle size interactions. The random effects included run, replicate within run and the four-way interaction of run with all the fixed effects. Data for both experiments were analyzed as a factorial set of variables, with the fixed effects for each experiment designated as factors. The least square means were calculated for DMD, total gas and CH₄ production, pH, VFA and NH₃-N. The control means was designated as mean point reference for each parameter measured, as this treatment consisted of substrate only with no biochar included. Biochar treatment means were then subtracted from the control mean, with differences expressed as deviations that allowed for factorial analysis and comparisons of each mean deviations of each biochar treatment at specific inclusion levels (Exp. 1 and 2) and particle size (Exp. 2). Orthogonal polynomial contrasts were performed to test parameter responses (linear, quadratic) to biochar inclusion and to directly compare each biochar treatment with the control. The type I error rate for hypothesis testing was 0.05 (P values ≤ 0.05 were considered significant).

Results

Interaction of fixed effects in both Exps. 1 and 2 were not presented in this section as there were no significant interactions observed in the measured DMD, gas and associated ruminal fermentation parameters; thus, only the main effects were presented in the tables and discussed hereafter in further detail.

Dry matter disappearance

In Exp. 1, biochar inclusion affected DMD ($P < 0.01$; Table 2), with the CP001 and CP014 lower than the other biochar products. Furthermore, a negative linear response ($P < 0.01$; Table 3) was observed with increasing biochar inclusion level.

In Exp. 2, biochar inclusion did not affect DMD regardless of biochar product ($P = 0.0.34$; Table 4), biochar inclusion level ($P = 0.23$; Table 5) or biochar particle size ($P = 0.72$; Table 6). There were no linear nor quadratic responses ($P \geq 0.44$; Table 6) observed for biochar particle size.

Total gas and CH₄ production

In Exp. 1, relative to the control, biochar product did not affect ($P \geq 0.21$; Table 2) total gas production expressed in mL/g DM incubated or mL/g DMD. Biochar inclusion level also did not affect total gas production ($P \geq 0.26$) nor result in linear or quadratic responses ($P \geq 0.17$; Table 3). Methane, expressed in mL/g DM incubated, mL/g DMD, and as percent of total gas production was not affected by biochar regardless of product ($P \geq 0.10$; Table 2). Biochar inclusion level did not affect ($P \geq 0.15$) total CH₄ produced irrespective of unit of expression with no linear or quadratic responses ($P \geq 0.15$; Table 3).

In Exp. 2, compared to the control, biochar product did not affect ($P \geq 0.41$; Table 4) total gas production irrespective of unit regardless of any of the inclusion levels ($P \geq 0.31$; Table 5). Additionally, biochar particle size did not affect total gas production ($P \geq 0.28$; Table 6) Biochar regardless of product, did not affect ($P \geq 0.70$; Table 4) CH₄ production irrespective of the unit of expression. Biochar inclusion level ($P \geq 0.21$; Table 5) and biochar particle size ($P \geq 0.19$; Table 6) did not affect CH₄ production (Table 7), with no linear or quadratic responses for both total gas and CH₄ production ($P \geq 0.10$; Table 6).

Ruminal fermentation parameters

In Exp. 1, addition of biochar did not affect ruminal pH ($P = 0.24$; Table 2) nor did biochar inclusion level ($P = 0.31$; Table 3). Total VFA concentration was not affected by biochar product ($P = 0.22$; Table 2) nor inclusion level ($P = 0.31$; Table 3). Additionally, individual VFA concentrations (acetate, butyrate and propionate), BCVFA and A:P ratios as well as the concentrations of $\text{NH}_3\text{-N}$ (mg/dL) were not affected by biochar product ($P \geq 0.12$; Table 2) nor inclusion level ($P \geq 0.21$; Table 3). No linear or quadratic effects ($P \geq 0.10$) were observed with regards to biochar inclusion level.

Similar results were observed in Exp. 2 as biochar product ($P \geq 0.11$; Table 4) nor inclusion level ($P \geq 0.32$) did not have an effect on total and individual VFA concentrations. Concentration of BCVFA and A:P ratios were not affected by biochar product ($P \geq 0.29$; Table 4) nor inclusion level ($P \geq 0.31$; Table 5). The same trend was observed as biochar product ($P = 0.68$; Table 4) and inclusion level ($P = 0.14$; Table 5) did not affect the concentration of $\text{NH}_3\text{-N}$. Biochar particle size had no effect on all the ruminal fermentation parameters measured ($P \geq 0.18$; Table 6) with no linear nor quadratic response ($P \geq 0.21$).

Discussion

Dry matter disappearance

In this study, biochar product and inclusion level decreased DMD in Exp. 1, whereas no response was observed in Exp. 2. Moreover, in Exp. 1 a negative linear decrease was observed in DMD when the level of biochar inclusion increased, regardless of which biochar product. The biochar products in Exp. 1 were included at 4.5, 13.5 and 22.5 % diet DM (0.5, 1.5, and 2.5 mg/ml of vial inoculum, respectively), which were higher than the inclusion levels in Exp. 2 (2.25 and

4.5 % diet DM; 0.25 and 0.5 mg/ml of inoculum respectively) which may explain the difference in responses. Biochar is relatively indigestible and may have impeded colonization of fibrolytic and cellulolytic bacteria involved in feed digestion (Schmidt and Noack 2000), especially at higher levels of inclusion. The results in Exp. 1 are supported by the findings of O'Reilly et al. (2021) as substrate DMD (vegetable mixed ration, maize silage and winter pasture) significantly decreased when wood-based biochar was supplemented at increasing levels of inclusion (0.5, 1.0, 2.0, 4.0 % DM). Additionally, two of the inclusion levels in Exp. 1 were also higher than that in the study of Hansen et al. (2012) who reported numerical but non significant reductions (2-12%) in DMD of biochar supplemented hay mixed ration *in vitro* evaluating the effects of wood-based and activated biochar included at 9 % of diet DM. Saleem et al. (2018) observed a significant linear increase in DMD of a barley silage-based TMR in an *in vitro* RUSITEC system with increasing, but at lower levels (0.5, 1.0, 2.0 % diet DM) of CP016 than the current experiments, however the mechanism for this response is unclear. Notwithstanding, the lack of DMD response in Exp. 2 agreed with Teoh et al. (2019) where the DMD of oaten pasture/maize silage/concentrate TMR was not affected by biochar inclusion. Also, in Exp. 2, biochar particle size did not affect DMD, suggesting that it does not influence ruminal fermentation kinetics. McFarlane et al. (2017), although observing similar results where biochar regardless of product did not affect forage digestibility relative to the control, reported significantly higher DMD in samples supplemented with smaller particle size biochar (< 0.178 mm) compared to larger particle size biochar (> 0.178 mm). These authors suggested that smaller particle size may have less adverse impacts on digestibility than larger sized biochar, owing to increasing surface area, which was in agreement with increased gas production in their *in vitro* study. However, in the current experiments the relationship of the original particle size distribution (e.g. CP001; D 0.1, 0.25; D 0.5, 0.95; D 0.9, 1.85) to biochar

particle size generated for the analysis (Exp. 1; 1.0 mm; Exp. 2; < 0.5, 0.5-2.0, > 2.0 mm) is unclear as the former may have been altered upon processing into the particular particle sizes for the study. Overall, the indigestible nature and high inclusion levels of biochar may have also contributed to the negative response in DMD of the TMR in Exp. 1, and not in Exp. 2.

The differences in DMD between coconut (CP001, CP014) and pine wood-based biochars (CP002, CP015, CP016, CP023 and CP024) in Exp. 1 may be attributed to several factors, such as the differing biomass feedstocks, resulting in the differences in C and ash content, as well as the physiochemical characteristics including the overall structure and porosity of the biochars (McFarlane et al. 2017; Gonzaga et al. 2018); however, in the current study, it is difficult to fully assess these differences as the chemical characteristics of the biochars evaluated were limited. In terms of chemical characteristics, the C content, which ranged from 71.1 to 81.6 % DM did not have varying effects on DMD. Overall, C content does not seem to be a factor influencing biochar effects on feed digestibility, regardless of amount (% DM) as observed in the inconsistencies in DMD effects in studies that supplemented biochar products with either low (10.0 % DM; Teoh et al. 2019), intermediate (76.9 % DM, Saleem et al. 2018; 58.0 % DM, O'Reilly et al. 2021) or high (91.2 % DM; Hansen et al. 2012) C content. Additionally, in the current study, the biochar pHs ranged from slightly acidic to neutral (4.9 to 7.3) and also did not have an effect on DMD. Teoh et al. (2019) and O'Reilly et al. (2021) used alkaline biochars (pH 8.2 and 8.4, respectively) and observed a range of effects, including significant reductions in DMD to no effect at all respectively, whereas Saleem et al. (2018) observed increased substrate DMD upon the addition of acidic biochar (pH 4.8). It has been reported that biochar, especially alkaline types, may exhibit buffering capacity in anaerobic digestion of complex organic wastes by acting as a temporary electron acceptor and promoting the oxidation of butyrate, preventing decreases in pH (Wang et al. 2018);

however, this was not the case in the current study and several cited studies. Based on the disparity of the results in the current study and published literature it is difficult to ascertain whether a specific physiochemical property (i.e., C content, pH) can be a determining factor in the observed DMD effects as production technologies, pyrolysis conditions, biomass source and after production processing of biochars can be variable (O'Reilly et al. 2021).

Total gas and CH₄ production

In both experiments, total gas and CH₄ production were not affected by biochar regardless of product, inclusion level or particle size which is in agreement with several *in vitro* studies (Pereira et al. 2014; McFarlane et al. 2017; Cabeza et al. 2018; Teoh et al. 2019), who also reported that biochar, regardless of source/chemical/physical composition did not mitigate ruminal CH₄ emissions. Additionally, an *in vivo* study by Terry et al. (2019) showed that addition of a pine-based biochar (0, 0.5, 1 and 2 % diet DM) to a barley-silage based diet did not alter CH₄ production in beef heifers. It is well established that the disappearance of feed substrate in the rumen (and resultant VFA patterns) influences CH₄ production (Johnson and Johnson 1995; Winders et al. 2019). This is in agreement with the results of Exp. 2, with the absence of responses in DMD, total gas/CH₄ production or associated fermentation parameters. Notwithstanding, although a significant decrease in DMD was observed in Exp. 1 no decrease in total gas or CH₄ production was identified. This reduction in DMD was small (3.0 %) and thus might not have been of a sufficient biological magnitude to result in a reduction in gas production. Similarly, O'Reilly et al. (2021) reported no change in total gas production (mL/g DM) *in vitro* when biochar (0.5, 1.0, 2.0, 4.0 % diet DM) was included in a variety of substrates *in vitro*, which corresponded to a significant

decrease in DMD. In terms of particle size, differently sized biochar in Exp. 2 did not affect gas or CH₄ production.

The absence of an impact of source was also reported by McFarlane et al. (2017) with no differences in total gas production among biochar samples regardless of biomass source but observed higher gas production in samples supplemented with finer (< 0.178 mm) particle-sized biochar, which aligned with an increase in DMD. Pereira et al. (2014) also observed no differences in total gas production between ryegrass ensiled with four biochar products (pine wood chips and corn stover pyrolyzed at 350 vs 550°C) compared to control at three inclusion levels (0.5, 1.0 and 2.0 % diet DM). In terms of CH₄ production, these authors did not observe differences between the biochar and control diets. However, in contrast, Saleem et al. (2018) reported a significant reduction in CH₄ production (7.7-22.0 %, depending on unit of expression), with a concurrent linear increase in DMD with CP016 included in a silage-based diet in an artificial rumen system. The differences in the results of Saleem et al. (2018) and the current study are not fully understood and may be attributed to the experimental model (batch culture vs RUSITEC), biochar products, as well as inclusion levels.

Furthermore, in an *in vivo* study, Leng et al. (2012c) reported a 24.3% reduction of CH₄ with 0.6% diet DM inclusion of a rice hull-based biochar pyrolyzed at 900°C to southeast Asian cattle fed cassava roots and foliage. In that study, DM intake was unaffected by biochar inclusion, hence the reduction may be partially attributed to the hypothesis that biochar aids in the formation of methanotrophic consortia on the inert surfaces of biochar (Leng et al. 2014; Kammann et al. 2017). However, this hypothesis was not confirmed and maybe unlikely as methanotrophs are often absent or present in extremely low numbers in the rumen (Henderson et al. 2015; Parmar et al. 2015; Auffret et al. 2018). Biochar is also hypothesized (Leng et al. 2013; Kammann et al.

2017) to facilitate redox reactions between syntrophic (fermentative bacteria and methanogens) microbial populations resulting in improved microbial growth. The enhanced microbial growth is suggested to encourage biofilm formation and improve feed conversion efficiency, therefore decreasing CH₄ output per unit of feed (Leng et al. 2012a, b, c; Wu et al. 2016; Kammann et al. 2017). However, this may be unlikely as it is difficult to assess how a relative change in porosity would have an effect on the microbial activity and as differing pore volumes, surface areas and particle sizes in biochar elicited no effect on measured parameters in this study. This was further supported by electron microscopy analysis completed by Terry et al. (2019) comparing feed and biochar surface biofilms, and the metagenomic data analysis of ruminal microbiota in the biochar studies of Teoh et al. (2019) and Terry et al. (2019). These authors observed no overall significant improvements in ruminal fermentation regardless of shifts in select ruminal microbial groups.

Pyrolysis temperature can influence the overall physiochemical profile of biochar and its consequent effect on ruminal microbiota as observed in the reports of Leng et al. (2012a, b, c; 2013). The current experiments used biochar products pyrolyzed at temperatures ranging from 400 to 600 °C which was similar to Pereira et al. 2014 (350 and 550 °C) and Teoh et al. 2019 (650 °C) but lower than Leng et al. (2012a, b, c) who utilized products pyrolyzed at 900 °C. High pyrolysis temperatures (600 – 1000 °C) are reported to increase pore volumes, which is speculated to favor the gas sorption capacity of biochar (Lehmann and Joseph 2009) as well as aiding the establishment of alternative biofilms within the rumen (Leng et al. 2013).. Nevertheless, it is still unclear how biochar with increased porosity included in diets at relatively low levels can impact the ruminal microbiota and fermentation. Digestible sugars and available carbohydrates are also likely combusted in the pyrolysis step in biochar production, which making biochar indigestible and inert thus unmetabolizable in the rumen.

Ruminal fermentation parameters

The rumen fermenter fluid pH was not affected by the differing biochar products in either experiment and reflected the commonality of VFA concentrations across incubations. The absence of an effect on VFAs was in agreement with Pereira et al. (2014) also reported that molar proportions of VFAs were unaffected by biochar *in vitro*, regardless of source (pine wood or straw-based) or inclusion level (0, 2.1, 4.2, 8.1, 18.6 % diet DM) to hay silage-based diet. This was further confirmed by McFarlane et al. (2017) who observed no effect of biochar source (chestnut oak, yellow poplar and white pine) when included at 8.1 % of diet DM in an orchard grass-based diet, regardless of particle size (< 0.178 or > 0.178 mm).

The effect of biochar on $\text{NH}_3\text{-N}$ production is not definitive as studies have reported no effect (Pereira et al. 2014), a decrease (Cabeza et al. 2018) or an increase (Saleem et al. 2018). The latter authors suggested that this increase may be due to increased ruminal protein degradation, which was also evidenced by increased branched VFA concentrations in their study. Ruminal $\text{NH}_3\text{-N}$ concentration is an indicator of the efficiency of conversion of dietary N to microbial N (Firkins et al. 2007) and it is suggested that biochar can enhance AA deamination (Saleem et al. 2018). Cabeza et al. (2018), who observed a decrease in $\text{NH}_3\text{-N}$, suggested that biochar (1.2 -12 % diet DM) may have adsorbed the $\text{NH}_3\text{-N}$, a phenomenon also reported as biochar prevents $\text{NH}_3\text{-N}$ leaching in soils (Ding et al. 2010). This is not clear, however, as biochar, being enriched with recalcitrant C may have different effects on soils compared to the rumen. Biochar amendment may be advantageous in soils as C:N ratios are improved, with NH_3 volatilization being regulated via absorbing NH_4^+ , resulting in decrease in N_2O emissions and N leaching (Kamman et al. 2017). This might not be the case in the rumen as the residence time is much shorter and the synchronization of C and N is unlikely due to biochar's recalcitrance. Therefore, this, in addition

to the lack of response to DMD and VFA results, may have resulted in the non-response in NH_3 -N production.

Conclusions

Overall, the inclusion of biochar products differing in source, physical and chemical characteristics, as well as particle size did not affect DMD, total gas or CH_4 production or ruminal fermentation in a barley silage-based TMR. However, biochar supplemented at high inclusion levels may negatively impact DMD. In conclusion, in this *in vitro* study, biochar as a feed additive did not demonstrate potential to mitigate enteric CH_4 or improve DMD or ruminal fermentation of barley silage-based diets *in vitro*.

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Table 1. Source, chemical and physical characteristics of seven biochar products^z used in in vitro Exp 1 and 2.

Parameter	Biochar product						
	CP001	CP002	CP014	CP015	CP016	CP023	CP024
Source/biomass origin	Coconut	Pine	Coconut	Pine	Pine	Pine	Pine
Chemical characteristics							
Carbon, % DM	75.6	81.6	76.6	75.4	76.9	75.3	71.1
pH	6.3	5.8	5.0	4.9	4.9	7.6	7.3
Physical characteristics							
Bulk density, kg/m ³	706.0	310.0	606.0	262.0	287.0	122.0	140.0
Surface area, m ² /g	161.0	218.0	160.0	189.0	186.0	152.0	148.0
Pore volume, cc/g	6.45×10^{-2}	8.75×10^{-2}	6.52×10^{-2}	7.56×10^{-2}	7.36×10^{-2}	6.10×10^{-2}	6.00×10^{-2}
Particle size distribution ^y , mm							
D 0.1	0.25	0.15	0.29	0.29	1.60	0.85	0.73
D 0.5	0.95	1.25	0.47	0.50	4.30	1.95	1.75
D 0.9	1.85	2.95	0.73	0.83	5.95	3.00	3.15

^zMeasurements regarding physical and chemical characteristics conducted by Cool Planet[®]

^yParticle size distribution: where “D” is the diameter and the number (0.1, 0.5, 0.9) are probabilities of finding a particle size (undersized, average size, oversized in the batch) with the diameter D in a given volume; with D0.5 being the median particle size.

Table 2. Main effects of biochar product on DMD, total gas and CH₄ production and rumen fermentation parameters in Exp 1.

Parameter ^z	Control	Biochar product							SEM	P value
		CP001	CP002	CP014	CP015	CP016	CP023	CP024		
DMD, %	52.3	49.3a	52.4b	50.8a	51.9b	52.2b	51.6b	52.1b	1.34	0.003
Total gas production, mL/g DM incubated	186.1	188.3	184.0	186.6	184.0	184.8	186.0	189.1	3.82	0.43
Total gas production, mL/g DMD	189.6	191.6	188.8	188.4	188.6	187.9	188.0	190.0	1.87	0.21
CH ₄ , mL/g DM incubated	17.9	18.4	17.8	17.9	17.4	17.7	18.1	18.6	0.84	0.10
CH ₄ , mL/g DMD	18.2	18.5	18.1	18.0	17.5	18.1	18.2	18.2	0.62	0.33
CH ₄ , % of total gas production	9.6	9.6	9.6	9.8	9.6	9.6	9.7	9.7	0.23	0.73
pH	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	0.02	0.24
Total VFA, mmol/L	75.0	70.4	72.5	80.7	75.2	78.9	67.7	80.0	12.35	0.22
Acetate	45.2	41.4	42.7	48.3	44.2	47.9	40.5	47.8	9.14	0.48
Propionate	16.5	16.2	16.7	18.3	16.8	17.7	14.3	18.2	2.22	0.12
Butyrate	10.5	10.1	10.5	11.3	11.5	10.6	10.3	11.2	2.07	0.66
BCVFA ^y	2.9	2.7	2.7	2.9	2.7	2.7	2.5	2.7	0.54	0.80
A: P ^x	2.8	2.8	2.8	2.6	2.6	2.7	2.8	2.7	0.34	0.44
NH ₃ -N, mg/dL	11.7	10.1	9.8	10.7	10.8	10.4	10.6	10.2	0.29	0.17

^zExp. 1 data were analyzed as a factorial set of treatments, with factors biochar product and inclusion level. Interaction was not significant and main effects of biochar product is shown here. The control means was used as a point of reference, from which biochar means were subtracted to calculate for control and treatment differences expressed as deviations. Mean deviations for each treatment were thereafter calculated through factorial analysis and were added to the control mean to report main effect means relative to the control as shown here.

^yBranched VFA (Isovalerate + Isobutyrate)

^xAcetate to propionate ratio

Table 3. Main effects of biochar inclusion level on DMD, total gas and CH₄ production and rumen fermentation parameters in Exp 1.

Parameter ^z	Biochar inclusion level				SEM	P value	Response	
	% diet DM						P value	
	Control (0)	4.5	13.5	22.5			Linear	Quadratic
DMD, %	52.3	52.0	51.7	50.4	1.12	0.001	0.002	0.074
Total gas production, mL/g DM incubated	186.1	184.6	187.7	186.7	1.76	0.26	0.17	0.37
Total gas production, mL/g DMD	189.6	187.8	188.6	188.7	1.98	0.86	0.62	0.81
CH ₄ , mL/g DM incubated	17.9	17.7	18.6	18.2	0.41	0.15	0.27	0.81
CH ₄ , mL/g DMD	18.2	17.8	18.3	18.0	0.32	0.16	0.52	0.63
CH ₄ , % total gas production	9.6	9.6	9.7	9.7	0.27	0.68	0.15	0.68
pH	6.3	6.3	6.3	6.3	0.02	0.31	0.20	0.82
Total VFA, mmol/L	75.0	72.0	77.9	75.6	11.47	0.30	0.69	0.14
Acetate	45.2	41.9	47.3	44.9	8.71	0.25	0.35	0.17
Propionate	16.5	16.2	16.9	18.0	2.02	0.29	0.12	0.82
Butyrate	10.5	11.2	11.0	10.2	1.99	0.27	0.14	0.57
BCVFA ^y	2.9	2.8	2.7	2.5	0.53	0.21	0.10	0.56
A: P ^x	2.8	2.8	2.7	2.7	0.35	0.38	0.20	0.57
NH ₃ -N, mg/dL	11.7	10.3	10.6	10.2	0.90	0.22	0.60	0.10

^zExp. 1 data were analyzed as a factorial set of treatments, with factors biochar product and inclusion level. Interaction was not significant and main effects of biochar inclusion level is shown here. The control means was used as a point of reference, where biochar means were subtracted to calculate for differences expressed as deviations. Mean deviations for each treatment were thereafter calculated through factorial analysis and were added to the control mean to report main effect means relative to the control as shown here.

^yBranched VFA (Isovalerate + Isobutyrate)

^xAcetate to propionate ratio

Table 4. Main effects of biochar product on DMD, total gas and CH₄ production and rumen fermentation parameters in Exp 2.

Parameter ^z	Biochar product				SEM	P value
	Control	CP002	CP016	CP023		
DMD, %	55.4	55.7	56.2	56.1	0.84	0.34
Total gas production, mL/g DM incubated	209.5	209.9	207.8	206.8	6.97	0.56
Total gas production, mL/g DMD	225.3	228.8	229.3	226.4	7.79	0.41
CH ₄ , mL/g DM incubated	25.9	25.9	25.7	25.6	0.80	0.70
CH ₄ , mL/g DMD	27.9	28.3	28.2	28.2	0.86	0.95
CH ₄ , % total gas production	12.4	12.4	12.3	12.4	0.28	0.77
pH	6.0	6.0	6.1	6.1	0.01	0.63
Total VFA, mmol/L	121.5	123.9	121.2	120.3	8.58	0.84
Acetate	83.7	86.0	83.7	84.2	4.01	0.52
Propionate	23.3	23.4	23.1	22.4	0.81	0.17
Butyrate	14.5	14.6	14.4	13.7	0.52	0.11
BCVFA ^y	6.5	6.4	6.2	6.3	0.26	0.35
A: P ^x	3.6	3.8	3.7	3.7	0.10	0.29
NH ₃ -N, mg/dL	38.1	39.0	38.7	38.3	1.88	0.68

^zExp. 2 data were analyzed as a factorial set of treatments, with factors biochar product, inclusion level and particle size. Interaction was not significant and main effects of biochar product is shown here. The control means was used as a point of reference, where biochar means were subtracted to calculate for differences expressed as deviations. Mean deviations for each treatment were thereafter calculated through factorial analysis and were added to the control mean to report main effect means relative to the control as shown here.

^yBranched VFA (Isovalerate + Isobutyrate)

^xAcetate to propionate ratio

Table 5. Main effects of biochar inclusion level on DMD, total gas and CH₄ production and rumen fermentation parameters in Exp 2.

Parameter ^z	Biochar inclusion level			SEM	<i>P</i> value
	% diet DM				
	Control (0)	2.3	4.5		
DMD, %	55.4	55.8	56.1	0.64	0.23
Total gas production, mL/g DM incubated	209.5	207.6	208.7	6.97	0.63
Total gas production, mL/g DMD	225.3	226.1	228.3	7.79	0.31
CH ₄ , mL/g DM incubated	25.9	25.7	25.8	0.75	0.64
CH ₄ , mL/g DMD	27.9	28.0	28.4	0.75	0.21
CH ₄ , % total gas production	12.4	12.4	12.3	0.25	0.67
pH	6.0	6.1	6.1	0.01	0.42
Total VFA, mmol/L	121.5	122.6	121.2	8.17	0.88
Acetate	83.7	85.5	83.8	3.92	0.32
Propionate	23.3	23.0	23.0	0.77	0.99
Butyrate	14.5	14.1	14.5	0.49	0.53
BCVFA ^y	6.5	6.2	6.3	0.27	0.61
A: P ^x	3.6	3.7	3.7	0.08	0.31
NH ₃ -N concentration, mg/dL	38.1	38.2	39.1	1.86	0.14

^zExp. 2 data were analyzed as a factorial set of treatments, with factors biochar product, inclusion level and particle size. Interaction was not significant and main effects of biochar level of inclusion is shown here. The control means was used as a point of reference, where biochar means were subtracted to calculate for differences expressed as deviations. Mean deviations for each treatment were thereafter calculated through factorial analysis and were added to the control mean to report main effect means relative to the control as shown here.

^yBranched VFA (Isovalerate + Isobutyrate)

^xAcetate to propionate ratio

Table 6. Main effects of biochar particle size on measured DMD, total gas and CH₄ production and rumen fermentation parameters in Exp 2.

Parameter ^z	Biochar particle size				SEM	P value	Response	
	Control	< 0.5	0.5-2	> 2			Linear	Quadratic
DMD, %	55.4	55.9	56.1	56.2	0.61	0.72	0.44	0.82
Total gas production, mL/g DM incubated	209.5	208.7	206.3	209.5	6.97	0.51	0.80	0.26
Total gas production, mL/g DMD	225.3	227.7	226.6	230.3	7.79	0.28	0.28	0.24
CH ₄ , mL/g DM incubated	25.9	25.7	25.5	26.0	0.84	0.49	0.78	0.25
CH ₄ , mL/g DMD	27.9	28.0	28.0	28.6	0.78	0.19	0.93	0.50
CH ₄ , % total gas production	12.4	12.4	12.3	12.4	0.28	0.23	0.10	0.70
pH	6.0	6.1	6.1	6.1	0.01	0.90	0.75	0.74
Total VFA, mmol/L	121.5	119.9	121.3	124.4	8.58	0.57	0.75	0.32
Acetate	83.7	83.0	84.4	86.6	4.01	0.23	0.81	0.82
Propionate	23.3	22.8	22.8	23.3	0.81	0.67	0.46	0.60
Butyrate	14.5	14.1	14.2	14.5	0.51	0.65	0.39	0.74
BCVFA ^x	6.5	6.2	6.2	6.4	0.28	0.28	0.21	0.32
A: P ^w	3.6	3.6	3.7	3.7	0.09	0.18	0.64	0.99
NH ₃ -N, mg/dL	38.1	38.3	38.8	38.9	1.89	0.72	0.45	0.77

^zExp. 2 data were analyzed as a factorial set of treatments, with factors biochar product, inclusion level and particle size. Interaction was not significant and main effects of biochar particle size is shown here. The control means was used as a point of reference, where biochar means were subtracted to calculate for differences expressed as deviations. Mean deviations for each treatment were thereafter calculated through factorial analysis and were added to the control mean to report main effect means relative to the control as shown here.

^yBranched VFA (Isovalerate + Isobutyrate)

^xAcetate to propionate ratio