The Effects of Biochar and Fat Supplementation on Microbial Fermentation in Batch Cultures

Kaci Way
Department of Animal Sciences
The Ohio State University
Research Thesis
Advisor: Dr. Jeffrey Firkins
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Abstract:

Biochar is a byproduct formed by burning green waste for carbon sequestering in a process called pyrolysis. This product can be used as a soil amendment to benefit plant yield. It has also been used as a supplement for cattle, though benefits in ruminants are still being explored. Hansen et al. (2012) noted a numerical decrease in methane production in vitro. Ruminants erupt methane as a hydrogen sink in the reduced rumen environment. Without a way to remove hydrogen, the microbial ecosystem cannot function normally. Polyunsaturated fatty acids have been used to decrease methane production; however, they often depress NDF digestibility. Therefore, more research is necessary to confirm that decreasing methane production with biochar does not also result from depressing neutral detergent fiber (NDF) digestibility, a major component of dairy cattle diets. The objectives of this study were to observe NDF disappearance (NDFD), volatile fatty acid (VFA) production, and methane gas output with supplementation of a biochar product in rumen fluid batch cultures. The treatments (Trt) were biochar (BC) or biochar bolus (BCB, biochar with electrolytes). The diet provided was a high forage (HF) diet with concentrate pellets (33.3%), orchard grass (44.4%), alfalfa (22.2%), and either no supplemented fat or 3% dry matter (DM) as corn oil (CO). The BC and BCB were dosed (Inc) at either 0, 1, 2 or 4% of total DM. Separately, four round bottom flasks were used for gas production measurements because smaller culture tubes would not produce enough gas volume. The flasks were fed either HF or HF with BC at 2%. Data were analyzed utilizing PROC MIXED (v. 9.4, SAS Institute 2015) with the fixed effects of Trt, CO, Inc, and their interactions. The random effects were run and order of inoculation. BC did not decrease NDFD and with 2% - CO and 1% + CO NDFD increased. BCB also did not decrease NDFD and with 1% - CO, 4% - CO, and 1% + CO NDFD increased (P = 0.07, Trt*CO*Inc). For total VFA production, BC increased the concentration with 2% - CO, 4% - CO, and 4% + CO. BCB also increased total VFA with 4% - CO and 4% + CO (P = 0.02, Trt*CO*Inc). Although methane gas production was not significant, there was numerical reduction of 23.08 mg produced in 24 hours (P = 0.16). Methane (g/kg NDFD) decreased (P = 0.022) by 17.21 g/kg NDFD. A numerical decrease (P = 0.23) of 0.10 mg/d was also seen in hydrogen gas production. Therefore, BC could reduce methane output without depressing NDFD and VFA when implemented as a feed additive. With the current stress on agricultural practices to decrease environmental impacts, feeding biochar as a methane mitigation strategy could be crucial to the dairy industry while simultaneously utilizing a waste product.

Introduction:

Over the past few years, there has been a growing concern with the amount of greenhouse gases that have been emitted into the environment, with agriculture at the forefront. As a result of this, focus turns to the dairy industry and methane emissions. According to the EPA, methane (CH_4) makes up approximately 10.2% of greenhouse gas emission in the United States, with livestock responsible for 9% due to manure management, and 27% from enteric fermentation ("Overview of Greenhouse Gases"). Although livestock make up a relatively small percentage of greenhouse gases when compared to other industries, there is room for improvement and a growing demand for more environmentally conscious farming practices. For example, the state of California is in the process of reducing methane emissions by 40% from the data collected in 2013 with the goal set for 2030. As this deadline quickly approaches, new dietary supplementations, such as biochar, are being investigated.

Corn oil and PUFA's have been used as a hydrogen sink in the reduced rumen environment; however, these have negative effects on rumen function. Oil supplementation, especially those abundant in PUFA, can decrease fiber degradation by inhibiting ruminal protozoa (Zhang et al., 2019a). However, other studies have shown that, despite corn oil's ability to serve as a hydrogen sink, it may also negatively affect nutrient and fiber digestibility (McGinn et al., 2004). The implementation of PUFA in the diet have also reduced milk fat production, known as milk fat depression (MFD). This is due to the altering of rumen biohydrogenation of dietary polyunsaturated fatty acids, which leads to the synthesis of fatty acid intermediates that inhibit milk fat production (Bauman and Griinari, 2003).

A recent study has shown that biochar can stimulate the anaerobic oxidation of CH_4 by anaerobic hydrogenotrophic archaea, meaning that biochar could serve as a CH_4 mitigation strategy in environments such as the rumen (Zhang et al., 2019b). Biochar is a byproduct formed by burning green waste for carbon sequestering in a process called pyrolysis. This product can be used as a soil amendment to benefit plant yield, and it has also been used to supplement cattle, though benefits in ruminants are still being explored (Joseph et al., 2015). There is limited research available on biochar implementation in ruminant diets; however, there are multiple sources that show a reduction in methane gas production, without any apparent decrease in digestibility. A study by Hansen et al. (2012) looked at the effects of biochar on in vitro rumen methane production. The experiment used filter bags contained feed additives, along with three different biochar samples that were dosed at 9% of the feed dry matter. The experiment showed an improvement in methane production, although it was not significant. Numerically, there was between an 11% and 17% decrease in methane output across the three samples of biochar when compared to the control (Hansen et al., 2012). This information can prove to be supportive of the experiment that is to be performed in this study because other studies have reported a methane decrease. The Hansen et al. (2012) experiment also showed that NDF was not greatly impacted by the implementation of biochar in the diet, but more information is needed when it comes to this factor.

Another study on biochar viewed the effects biochar produced from different biomass sources, methane production and ammonia concentrations on an in vitro model. Different biochar inclusion rates were used, but no difference was reported (Cabeza et al., 2018). This is important to recognize because the optimum dosage of biochar has yet to be determined. There were no significant differences in methane emission. Varying degrees of methane reduction have been reported. Propionate and butyrate were reduced when biochar was implemented, and no negative effects on ammonia were shown. This means that biochar could be an effective supplement and it could be safely used on pastures and soil. Therefore, the objective of the current study is to observe NDF disappearance, volatile fatty acid (VFA) production, and methane gas output with supplementation of a biochar product in rumen fluid batch cultures.

Methods:

This experiment was conducted using a rumen fluid batch culture with 4 round bottom flasks and 72 culture tubes containing different diets, along with blanks. A total of two runs were conducted for this study. Preparation began a week before the batch culture was initiated. First, culture tubes were numbered (1-72) and were prepared by weighing out each feed ingredient. The culture tubes contained a total of 0.5 g DM. The high forage diet used is described in Table 1 consisted of a concentrate pelleted feed (33.3%) along with orchard grass (44.4%), alfalfa pellets (22.2%), and either no supplemented fat or 3% dry matter (DM) as corn oil (CO). A high forage diet was used to increase methane production to ensure there was enough for sample collection. The treatments were biochar (BC) and biochar bolus (BCB, biochar + electrolytes). Either product was dosed at 0%, 1%, 2% and 4% DMI. Previous studies have included biochar as 1-2% of DMI, but 4% inclusion was used to investigate whether there were negative effects at higher doses. Biochar bolus was included in this model because it is already used as a treatment in dairy cattle, whereas biochar would be implemented as a feed additive. At each level, 4 tubes had 3% DM as additional supplemental fat and 4 tubes did not. Four round-bottom flasks were also used to capture methane gas produced, containing a total of 10 g DM. The flasks were either dosed with BC at 2% inclusion rate or no treatment. Round bottom flasks were used because smaller culture tubes would not produce enough gas volume to measure methane production.

Table 1. Diet and nutrients supplied to batch culture and round bottom flasks.

Ingredient (% DM)[1]	High Forage	High Forage + Corn Oil (CO)
Alfalfa	22.22	22.22
Orchard grass	44.44	44.44
Corn grain	22.88	15.94
Corn starch	0.00	5.00
Dicalcium phosphate	0.20	0.20
Magnesium oxide	0.10	1.40
Selenium 200	0.14	0.14
Soybean hulls	2.76	0.00
Soybean meal	5.22	6.92
TM supplement	0.50	0.50
Vitamin (A, D, E)	0.13	0.13
Fat - calcium soaps	1.40	1.40
Fat - vegetable oil	0.00	3.00
Diet Composition (%)		
DM	92.40	92.69
NDF	38.83	38.17
CP	14.89	14.77
RUP[2]	5.40	5.33
RDP[2]	9.60	9.67
Fat[2]	2.97	5.40
Starch	16.60	16.56

[1] Diet was fed as ground alfalfa and orchard grass pellets, the remaining ingredients listed were mixed into a concentrate pellet that was ground before adding to the batch culture tubes or flasks

[2] Values are predicted from book values

Next, the buffer solution was prepared 24 hours before starting the batch culture. The media solution consisted of 2 L of distilled H_2O, 5 mL of a micromineral solution (30 g $CaCl_2*2H_2O$, 8 g $FeCl_3*6H_2O$, 10 g $MnCl_2*4H_2O$, 1 g $CoCl_2*6H_2O$, 100 mL distilled H_2O), 1 L of a macromineral solution (11.4 g Na_2HPO_4, 12.4 g KH_2PO_4, 1.2 g Mg_2SO_4, 2 L distilled H_2O), 1 L of a rumen buffer solution (78.94 g $NaHCO_3$, 2 L distilled H_2O), and 5 mL of 0.1% resazurin as an indicator of reduction. This was bubbled with CO_2 continuously for 24 hours. It was used to help maintain pH within the test tubes and round bottom flasks.

On the morning of the initiation of the batch culture, 250 mL of the reducing media solution (3.125 g L- Cysteine $HCl*H_2O$, 20mL 1N NaOH, 3.125 g Na_2S*9H_2O, 475 mL reduced distilled H_2O) was added to the media solution while rumen fluid was collected from a cannulated cow. This was done by squeezing rumen contents in a cheese cloth and collecting the rumen fluid in a funnel over a 250 mL container. Four containers were filled, and these were placed in a cooler with 39 °C water. This maintained the temperature of the rumen fluid until it was taken back to the lab. Next, the rumen fluid was placed in a blender to ensure that any large

contents that got through the cheese cloth were pureed to a small particle size and filtered through cheesecloth. The rumen fluid was then added to a beaker with CO_2 gas to maintain the anaerobic environment. Prepared buffer solution was added to a large, rectangular shaped container and placed into a water bath at 39 °C with the shaker on. Rumen fluid was added in a ratio of 1-part rumen fluid to 3-parts buffer. A CO_2 gas line with a bubbler was added to the mixture in order to maintain the anaerobic environment. In a random order, batch culture tubes were dosed with 30 mL of combined rumen fluid/buffer solution every 2-3 minutes while the rumen fluid and buffer was continuously being mixed. Simultaneously, CO_2 was flowing into the culture tubes. A stopper was placed on each test tube with a one-way valve that releases pressure out of the tube but maintains an anaerobic environment inside of the tube. The main purpose of the culture tubes was to look at how corn oil and biochar affected nutrient digestibility, VFA production, and VFA profile. After the stopper was sealed on the culture tube, it was placed in a test tube rack in the incubator in the order of random selection. The round bottom flasks were also randomly inoculated in a random order with the batch culture tubes and dosed with 600 mL rumen fluid/buffer solution while simultaneously adding CO_2. A stopper with a one-way valve attached to a mylar balloon was placed on each round bottom flask. The volume of the balloon prior to incubation was measured by water displacement. A one-way valve ensured only the gas leaving the round bottom flask was captured in the mylar balloon without allowing pressure to increase in the flasks, and it maintained an anerobic environment. The round bottom flasks were then placed in an incubator at 39 °C for 24 hours.

The batch culture was also incubated for a 24-hour time period. After 24 hours, samples were collected from the culture tubes and round bottom flasks in the order they were dosed with rumen fluid/buffer solution. The mylar balloon were detached from the round bottom flasks, and the final volume of the balloon was measured by water displacement. Gas samples from the balloons were tested by injecting the gas collected through a Micro-Oxymax Respirometer (Columbus Instruments Inc., Columbus, OH), which measures methane and hydrogen concentration.

Neutral detergent fiber was also measured after drying the batch culture tubes at 50 °C for approximately 2 days in an oven. The dried samples were scraped into 500-mL beakers that were combined with NDF solution. Reflux racks and a filtering system were used, followed by weighing back the NDF residue from the analysis.

Volatile fatty acids were tested by obtaining a 5-mL sample from the test tubes and round bottom flasks. After 24 hrs., the tubes were then placed on ice to stop fermentation. The 5-mL subsamples were placed into a 15-mL test tube already prepared with 1 mL of 25% meta-phosphoric acid that was made fresh that morning. The tubes were capped and vortexed and allowed to settle for 20 min. After settling, a 2-mL sample was pipetted into a 2-mL microtube, and the remaining sample was stored in a 5-mL microtube at -20°C. The 2-mL microtubes were centrifuged at 5,000 x g for 15 min at 4°C. Without disturbing the pellet, 1.7 mL was pipetted to a new 2-mL microtube and stored at -20°C; the previous tube was disposed. After the samples were completely frozen (left in the freezer at least overnight), the 2-mL microtube was allowed

to thaw at room temperature. After thawing, 0.17 mL of 109.92 mM pivalic acid was added as the internal standard. The samples were vortexed and then centrifuged at 5,000 x g for 15 min at 4°C, and the supernatant was transferred to a new 2-mL microtube. The tubes were refrozen at -20°C (at least overnight), thawed at room temperature, vortexed, centrifuged at 5,000 x g for 15 min at 4°C, and the supernatant was transferred to a new 2-mL microtube. This step was repeated until there was no pellet after centrifuging. After there was no pellet remaining in the sample, an additional 2-mL microtube was prepared with 1 mL of distilled H_2O and 0.4-mL sample. After vertexing the 2 mL microtube, the pH of the sample was tested with litmus paper and balanced to a pH of 6-7 with adding 4 N potassium hydroxide (KOH). Each sample was vortexed to mix prior to confirming the pH was neutral. The exact volume of 4 N KOH added was recorded and used to calculate the final dilution of the sample. From the neutral sample, 1 mL was added to a 2 mL gas chromatography (GC) vial with 0.1 mL of 0.3 oxalic acid, which was considered in the final dilution calculation. The vials were then capped, vortexed, and stored at -20˚C until the samples could be analyzed.

The VFA samples were analyzed with a splitless HP5890 GC equipped with a flame ionize detector (FID) and a 23110-U glass packed column (Sigma Aldrich St. Louis, MO). Nitrogen, the carrier gas, had a flow rate of 0.4 mL/s. The FID supply was H_2 (flow rate 0.5 mL/s) and air (> 1 mL/s). The inlet was 150°C, the dectore was 180°C, and the initial temperature was 175°C. The initial temperature was held for 18 min, then increased to 195°C at 25°C/min and was held for 10 minutes. At the beginning of the run, a standard curve was derived using an external standard (ES), which contained known concentrations of the acetate, propionate, isobutyrate, butyrate, IS, 2-methylbutyrate, isovalerate, valerate, and caproate, was used to confirm linearity. After every 10 samples, an ES sample (8.06 mM acetate, 2.72 mM propionate, 0.28 mM isobutyrate, 2.06 mM butyrate, 9.97 mM IS, 0.52 mM 2-methylbutyrate, 0.41 mM isovalerate, 0.41 mM valerate, 0.54 mM caproate) was injected and used to calculate a response factor for each VFA peak. Between each injection, a sample of distilled H_2O was injected and ran through the same conditions to maintain the column and prevent carryover between samples. After the samples were ran, the peaks were integrated, and total VFA concentration and individual VFA concentrations were calculated utilizing the following equations.

$$\textbf{Relative Response Factor for Individual VFA } (\mathbf{RRF_{VFA}}) = \frac{\mathbf{Area_{VFA(ES)}}}{\mathbf{[VFA]_{ES}}} \times \frac{\mathbf{[IS]_{ES}}}{\mathbf{Area_{IS(ES)}}}$$

$\text{Area}_{\text{VFA(ES)}} = \text{Area of the individual VFA in the ES}$

$\text{Area}_{\text{IS(ES)}} = \text{Area of the IS in the ES}$

$[\text{VFA}]_{\text{ES}} = \text{Known concentration of the individual VFA in the ES}$

$[\text{IS}]_{\text{ES}} = \text{Known concentration of the IS in the ES}$

$$\textbf{Concentration of Individual VFA } (\mathbf{mM}) = \frac{\mathbf{Area_{VFA(sample)}}}{\mathbf{Area_{IS(sample)}}} \times \frac{\mathbf{1}}{\mathbf{RRF_{VFA}}} \times \mathbf{[IS]_{sample}} \times \mathbf{MF}$$

$\text{Area}_{\text{VFA(sample)}} = \text{Area of the indivdual VFA in the diluated sample}$

$\text{Area}_{\text{IS(sample)}}$ = Area of the IS in the diluated sample

$[\text{IS}]_{\text{sample}}$ = concentraiton of IS in the diluated sample

MF = multiplication factor to correct for the dilution of the sample with distilled H_2O, KOH, and oxalic acid

The effect of BC, BCB, and CO on NDF disappearance and VFA production and profile were analyzed with PROC MIXED (v. 9.4, SAS Institute 2015). The model used was $Y_{ijklm} = \mu + T_i + I_j + F_k + (T \times I)_{ij} + (I \times F)_{jk} + (T \times F \times I)_{ijk} + O_l + R_m + e_{ijklm}$ where Y_{ijklm} = response variable; μ = overall mean response; T_i = the fix effect of treatment (i = 1 or 2) where 1 = BC and 2 = BCB; I_j = the fix effect of treatment inclusion (j = 0, 1, 2, or 4) where 0 = 0% treatment inclusion, 1 = 1% treatment inclusion, 2 = 2% treatment inclusion, and 4 = 4% treatment inclusion; F_k = the fix effect of CO (k = 0 or 1) where 0 = no supplemented corn oil, and 1 = 3% DM addition as corn oil); $(T \times I)_{ij}$ = the interaction of treatment and inclusion; $(I \times F)_{jk}$ = the interaction of inclusion and CO; $(T \times F \times I)_{ijk}$ = the interaction of treatment, inclusion, and CO; O_l = the random effects of order of inoculum (l = 1-76); R_m = the random effect of run (m = 1 or 2); and e_{ijklm} = residual error.

The effect of BC on gas production with the round bottom flasks was analyzed with PROC MIXED (v. 9.4, SAS Institute 2015). The model used was $Y_{ilm} = \mu + I_i + O_l + R_m + e_{ilm}$ where Y_{ilm} = response variable, μ = overall mean response; I_j = the fix effect of inclusion (j = 0 or 2) where 0 = no supplement and 2 = BC at 2% inclusion, O_l = the random effects of order of inoculum (l = 1-76), R_m = the random effect of run (m = 1 or 2).

From statistical model evaluation, residuals were normally distributed and there was homogeneity of variance. Significant differences were at P values of ≤ 0.05, and tendencies were at P values $0.05 < P \leq 0.10$. All data were expressed as LSM with SE.

Results:

Shown in Table 1, the overall disappearance of NDF (NDFD) did not change ($P = 0.53$) with treatment; however, there was a three-way interaction between treatment with BC and BCB, fat supplementation and percent inclusion ($P=0.068$) for this measurement. As displayed in Chart 1, there was an increase ($P = 0.0073$) of NDF disappearance only at 2% inclusion of BC and no supplemental fat compared to the 0% - CO. With no supplemental fat and 1% or 4% inclusion of BCB, NDF disappearance increased ($P < 0.068$), with the greatest change occurring at the 1% dose. The inclusion of supplemental fat and BC increased ($P=0.065$) NDFD at 1% inclusion. When there was supplemental fat and 1% inclusion of BCB, NDFD increased ($P = 0.041$). Supplemental fat did not decrease NDFD and actually increased ($P < 0.10$) the measurement at 1%, 2% BC compared to BC – CO at the same inclusions. At the BCB 2% inclusion, CO increased ($P = 0.08$) NDFD.

There was a three-way interaction between treatment with BC and BCB, CO, and inclusion ($P = 0.015$) for total VFA concentration. As shown in Chart 2, when BC with no CO was dosed at 2% or 4%, total VFA (mM) increased ($P < 0.0055$) with the greatest improvement at the 2% compared to 0% – CO. Total VFA also increased ($P = 0.030$) at 4% inclusion with BCB - CO. With CO and 1% or 4% inclusion of BC, total VFA (mM) increased ($P < 0.054$). With CO and 4% inclusion of BCB, total VFA increased ($P = 0.028$) compared to 0% + CO. Supplemental CO increased ($P = 0.0002$) total VFA when added with BC 2% compared to 2% BC – CO.

Between BC and BCB, there were no differences ($P = 0.45$) in the acetate: propionate. There was an interaction between inclusion and CO ($P = 0.028$) for this measurement. When there was no supplemental fat, any inclusion level of BC and BCB increased ($P < 0.022$) the ratio by 0.16-0.26. With supplemental fat and 1% or 2% inclusion of BC or BCB, there was no change ($P > 0.19$) in the acetate: propionate, but at the 4% dose, the ratio increased ($P = 0.09$) by 0.12. Additional CO increased ($P = 0.022$) the ratio at 0% inclusion by 0.16, but it decreased ($P = 0.013$) at the 2% inclusion by 0.20.

Acetate molar proportion had no differences ($P = 0.30$) between BC and BCB treatment. There was an interaction between fat supplementation and percent inclusion ($P = 0.034$). When fat was supplemented and 1% or 4% inclusion BC or BCB was dosed, acetate increased ($P < 0.088$) by 1.7-2.7 mol/100 mol. However, with 2% BC or BCB inclusion, acetate molar proportion did not change ($P = 0.17$). At the 2% inclusion supplemental CO decreased ($P = 0.0089$) acetate by 2.16 mol/ 100 mol. Between BC and BCB, there were no differences ($P = 0.52$) in propionate. There was an interaction between percent inclusion and CO ($P = 0.026$). When there was no supplemental fat, any inclusion level of BC and BCB decreased ($P < 0.025$) propionate by 0.96-1.5 mol/100 mol. With supplemental fat, there were no changes ($P > 0.15$) with inclusion doses. Additionally, CO decreased ($P = 0.025$) the proportion of propionate at the 0% and 2% inclusion. Isobutyrate molar concentration had no significant main effects or interactions ($P > 0.28$). There were no differences ($P = 0.19$) between treatment with BC and BCB on mol/100 mol butyrate. However, there was an interaction between supplemented fat and

percent inclusion ($P = 0.095$). When no fat was supplemented, any inclusion level of BC and BCB decreased ($P < 0.088$) by 0.60-1.03 mol/ 100 mol. With fat supplementation, there were no changes ($P > 0.12$) at 1% or 2% inclusion of BC or BCB; however, with 4% inclusion of BC or BCB, butyrate decreased ($P = 0.0038$) by 1.07 mol/100 mol. Supplemental CO increased ($P < 0.10$) the molar proportion of butyrate at the 0% and 2% inclusion of BC or BCB. As a proportion of total VFA, 2-methylbutyrate with 1% or 4% inclusion with BC or BCB decreased ($P < 0.034$) by 0.035- 0.061 mol/ 100 mol compared to the batch culture tubes with 0% - CO. However, there were no changes ($P = 0.11$) with 2% inclusion of BC or BCB. There was also an interaction between treatment with BC and BCB and fat supplementation ($P = 0.021$). When no fat was supplemented, BCB decreased ($P = 0.061$) 2-methylbutyrate by 0.032 mol/100 mol compared to BC – CO. There were no changes ($P = 0.16$) between BC and BCB treatment with fat supplementation. Also, CO increased 2-methylbutyate molar proportion when added to BCB ($P = 0.021$) by 0.04 mol/ 100 mol. Isovalerate did not display any significant main effects or interactions. There was an interaction between fat supplementation and percent inclusion for valerate as a proportion of total VFA ($P = 0.031$). When there was no fat supplementation and 2% or 4% inclusion of BC or BCB, valerate decreased ($P < 0.016$) by 0.18- 0.19 mol/100 mol, whereas 1% inclusion had no change ($P = 0.23$) compared to 0% – CO. With fat supplementation and 1% or 4% inclusion with BC or BCB valerate decreased ($P < 0.0047$) by 0.147-0.15 mol/100 mol. However, there were no changes with 2% inclusion of BC or BCB ($P = 0.65$). Supplemental CO decreased ($P = 0.075$) valerate at the 1% and 2% dose of BC or BCB compared to 0% – CO. Additionally, there was an interaction between treatment with BC and BCB and fat supplementation. There were no differences ($P = 0.54$) between BC and BCB treatment when no fat was supplemented, and supplemental CO did not affect ($P > 0.13$) valerate with BC or BCB. With fat supplementation, BCB increased ($P = 0.064$) valerate by 0.068 mol/100 mol compared to BC + CO. There were no differences ($P = 0.32$) between treatment with BC and BCB on mol/100 mol caproate. As a proportion of total VFA, caproate with 1% or 4% inclusion with BC or BCB decreased ($P < 0.082$) by 0.03-0.05, but 2% inclusion had no change ($P = 0.21$). When fat was supplemented, caproate increased ($P < 0.001$) by 0.10 mol/100 mol.

Although methane gas production was not significant, there was numerical reduction of 23.08 mg produced in 24 hours ($P = 0.16$) with BC. Methane g/kg NDFD was estimated for the round bottom flasks using NDFD results of the batch culture tubes. This measurement decreased ($P = 0.022$) by 17.21 g/kg NDFD with 2% BC. A numerical decrease ($P = 0.23$) of 0.10 mg/d was also seen in hydrogen gas production with BC.

Table 1: Neutral detergent fiber (NDF), total volatile fatty acid (VFA), VFA profile, and gas production

	No Supplement		BioChar						BioChar Bolus						SEM	Significance (*P* value)						
	-CO	+CO	-CO			+CO			-CO			+CO										
	0%	0%	1%	2%	4%	1%	2%	4%	1%	2%	4%	1%	2%	4%		Trt[1]	CO[2]	Inc[3]	Trt *CO	Trt *Inc	CO *Inc	Trt*CO *Inc
Disappearance (%)																						
NDF	38.23	41.35	40.51	47.59	40.28	47.35	41.51	43.86	46.77	39.24	44.01	48.82	45.77	42.73	3.22	0.53	0.076	< 0.01	0.77	0.45	0.66	0.068
VFA (mM)																						
Total VFA	64.38	64.90	65.90	77.20	77.09	73.25	58.15	75.07	66.62	66.23	74.36	64.75	69.93	74.64	5.57	0.43	0.42	< 0.01	0.26	0.83	0.19	0.015
Acetate: Propionate	2.27	2.36	2.53	2.49	2.53	2.47	2.20	2.55	2.32	2.46	2.53	2.46	2.35	2.42	0.12	0.45	0.49	< 0.01	0.39	0.47	0.028	0.44
VFA (mol/100 mol)																						
Acetate	57.02	57.93	59.48	59.17	59.80	59.61	56.47	60.72	57.87	57.87	58.91	59.02	57.46	58.90	0.98	0.30	0.78	< 0.01	0.85	0.50	0.034	0.58
Propionate	25.14	24.55	23.46	23.74	23.59	24.14	25.60	23.83	24.91	24.08	23.75	23.95	24.67	24.27	0.67	0.52	0.37	0.011	0.12	0.57	0.026	0.42
Isobutyrate	0.62	0.62	0.63	0.59	0.62	0.64	0.56	0.60	0.58	0.62	0.57	0.59	0.62	0.58	0.61	0.54	0.89	0.60	0.71	0.28	0.97	0.94
Butyrate	12.21	11.74	11.47	11.44	11.07	10.76	12.33	10.08	11.75	11.51	11.28	11.48	12.00	11.22	0.48	0.19	0.31	< 0.01	0.55	0.41	0.095	0.67
2-methylbutyrate	0.80	0.82	0.80	0.79	0.78	0.78	0.76	0.74	0.75	0.76	0.72	0.78	0.82	0.78	0.032	0.75	0.28	< 0.01	0.021	0.77	0.96	0.45
Isovalerate	0.42	0.44	0.44	0.39	0.42	0.41	0.35	0.37	0.37	0.41	0.35	0.41	0.44	0.38	0.062	0.95	0.98	0.23	0.14	0.37	0.94	0.77
Valerate	1.77	1.75	1.70	1.68	1.61	1.57	1.76	1.54	1.73	1.62	1.55	1.66	1.80	1.67	0.07	0.37	0.62	0.081	< 0.01	0.80	0.031	0.60
Caproate	0.35	0.45	0.32	0.37	0.30	0.38	0.48	0.37	0.36	0.33	0.29	0.41	0.49	0.43	0.033	0.32	< 0.01	< 0.01	0.26	0.57	0.32	0.73
Gas Production																						
Methane (mg/d)	93.19	-	-	70.11	-	-	-	-	-	-	-	-	-	-	38.82	-	-	0.16	-	-	-	-
Methane g/kg NDF Disappeared[4]	47.27	-	-	30.06	-	-	-	-	-	-	-	-	-	-	3.36	-	-	0.022	-	-	-	-
Hydrogen (mg/d)	0.5905	-	-	0.4883	-	-	-	-	-	-	-	-	-	-	0.2063	-	-	0.2336	-	-	-	-

[1] Treatment (Trt) was either Biochar (BC) or BioChar Bolus (BCB)
[2] Corn oil (CO) was either +3% DM as CO or no additional supplemental corn oil
[3] Inclusion (Inc) was the dose of BC or BCB (0%, 1%, 2%, or 4% DM)
[4] Methane efficiency (g Methane/kg NDF Disappeared) was estimated utilizing the NDF disappearance (%) of the batch culture tubes within run for the NDF disappearance % of the round bottom flasks

Chart 1: None of the treatments decreased neutral detergent fiber disappearance (NDFD) and 1% and 2% inclusion display an increase in NDFD; ▪ = No Supplement, ▨ = No Supplement + Corn Oil (CO), ▪ = Biochar (BC), ▨ = BC + CO, ▪ = Biochar Bolus (BCB), and ▨ = BCB +CO (letters not shared between bars indicates a *P* value ≤ 0.10 between LSM and bars indicate ± SEM)

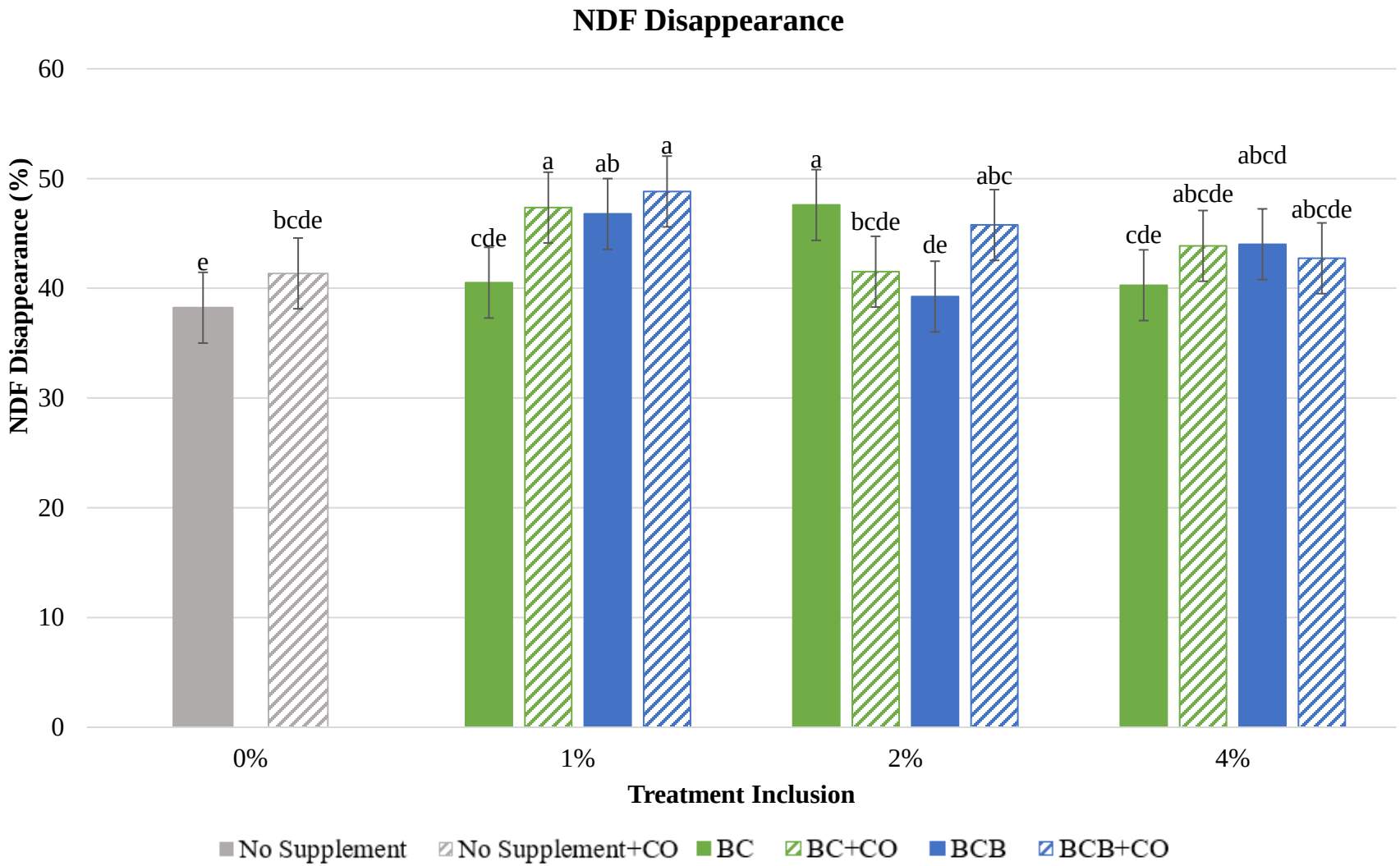
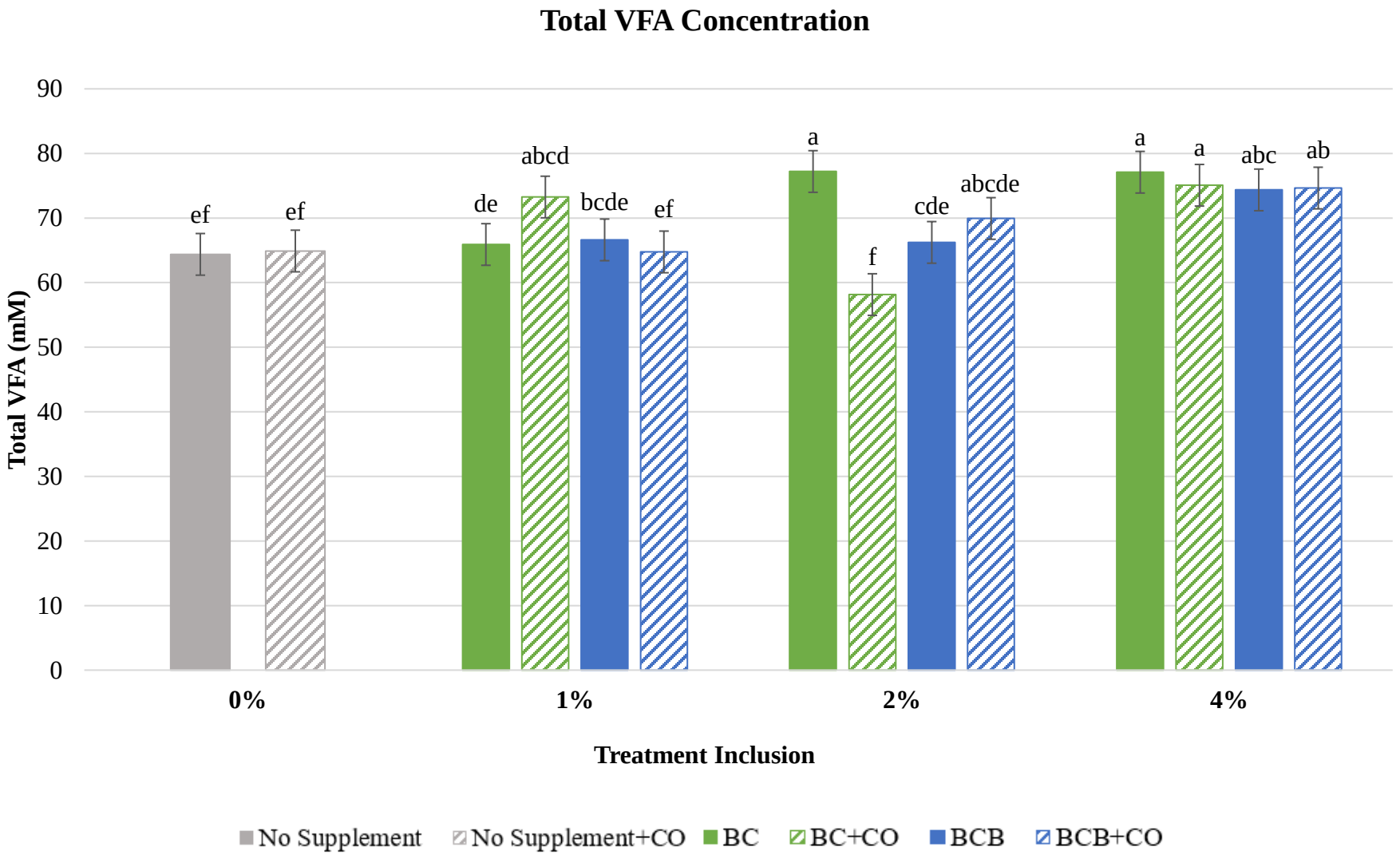


Chart 2: Total volatile fatty acid (mM) did not decrease with Biochar (BC), Biochar Bolus (BCB), or Corn Oil and 4% inclusion of BCB and BC increased total VFA; ■ = No Supplement, ▨ = No Supplement + CO ■ = BC ▨ = BC + CO, ■ = BCB and ▨ = BCB + CO (letters not shared between bars indicates a *P* value ≤ 0.10 between LSM and bars indicate ± SEM)



Discussion:

Previous research has shown that NDFD is not greatly impacted by biochar (BC) supplementation (Hansen et al., 2012). In accordance with the Hansen et al experiment, this study also displayed no negative effects of corn oil (CO) or BC on NDFD. In certain cases, there were even increases in NDFD. The addition of supplemental fat and BC increased NDFD at the 1% and 2% inclusions when compared to BC – CO. There was also an increase in NDFD with BCB 2% + CO compared to BCB 2% – CO. These results were similar with another study by Zang et al. (2019a), who observed the effects of corn oil supplementation on fermentation pathways in ruminants. In their study, CO supplementation had a tendency for increased NDFD. It is not certain as to why BC and BCB treatment displayed increases in NDFD and more research will need to be conducted to answer these questions.

As mentioned previously, the use of BC or BCB as a supplement in the ruminant diet is still being explored. In this study, total VFA and the proportion of VFA were evaluated after the usage of BC or BCB in a rumen fluid batch culture. In total, VFA production did not decrease with BC, BCB, or CO supplementation. In fact, BC increased the total VFA concentration with 2% and 4% percent inclusion without fat supplementation, when compared to 0% – CO. Total VFA also increased 4% inclusion with CO. There were also increases in total VFA concentration with BCB. These increases were with 4% inclusion without CO supplementation and 4% inclusion with CO supplementation, when compared to 0% inclusion with CO. Comparingly, when BC treatment was included in an experiment by Cabeza et al. (2018), there were no noted changes in total VFA production or acetate. Different percent inclusions and corn oil were not tested, which could account for the slight differences in this data. Supplemental CO also increased total VFA when it was added with BC 2% inclusion and compared to BC – CO. A total VFA concentration increase ($P = 0.027$) also occurred in the Zhang et al. (2019a) experiment. The study suggests that this increase occurred because acetate generation is thermodynamically favorable under low ruminal H2 partial pressure, which facilitates H_2 generation through acetate production.

In addition to increases in total VFA concentration, there was also an increase in acetate: propionate when fat was supplemented at 1% or 4% inclusion of BC or BCB. However, at 2% inclusion with fat supplementation, there were no changes in the acetate: propionate ratio. This ratio increase is primarily due to the increase in fiber digestibility. Although the acetate: propionate ratio is not included in other biochar studies such as the Zhang et al. (2019a) experiment also reported an increase in the ratio when CO was supplemented.

Acetate molar proportion had no differences between BC and BCB treatment; however, when fat was supplemented with 1% or 4% inclusion of BC or BCB, there was an increase in acetate by 1.7-2.7 mol/100 mol. This increase in acetate molar proportion differs from a previous study that compared different biomass sources as there were no changes in total VFA or acetic acid production during in vitro fermentation (Cabeza et al., 2018). One inconsistency in this study was when fat was supplemented with 2% inclusion, acetate decreased in molar proportion. It is possible that the decrease in acetate molar proportion were due to its release as an

intermediate during biochar-mediated microbial respiration and CO_2 production (Zhang et al., 2019b). In other words, this decrease could be a result of acetate utilization during the fermentation process.

There were changes in the propionate molar proportion with the supplementation of CO and percent inclusion. This molar proportion decreased with any percent inclusion of BC or BCB when no fat was supplemented. Similarly, in the Cabeza et al. (2018) experiment, the proportion of propionate decreased with the addition of biochar. However, when CO was supplemented, the proportion of propionate increased at the 0% and 2% inclusion of BC or BCB. This proportion most likely decreased due to increase in acetate molar proportion with 1% + CO and 2% + CO.

A previous study indicated a decrease in butyrate molar proportion with the utilization of biochar in a fermentation experiment (Cabeza et al., 2018). Butyrate also decreased in this experiment when no CO supplementation was included and at any inclusion level of BC and BCB. With fat supplementation, the molar proportion increased at 0% and 2% inclusion; however, there was a decrease with 4% inclusion of BC or BCB. Therefore, with the addition of CO, more biochar or biochar bolus was needed to cause a decrease in the butyrate molar proportion. Butyrate producing bacterial also have a role in biohydrogenation bacteria; thus, the addition of corn oil may cause and increase in this process which results in an increase in butyrate (Polan et al., 1964). This change could also be due to decreases in the proportion of acetate with 2% BC or BCB inclusion and increases in acetate with 1% or 4%; therefore, butyrate and acetate act inversely in some cases of biochar inclusion.

Isobutyrate, 2-methylbutyrate, isovalerate (branched-chain volatile fatty acids, BCVFA) and valerate have been shown to be growth promoting factors for rumen microbes, especially fiber digesting bacteria (Allison, 1963; Dehority, 1967; Robinson, 1968). There were no changes in isobutyrate and isovalerate with the current study, however, there were differences in 2-methylbutyrate and valerate. For 2-methylbutyrate, there were differences between treatment with BC and BCB. Biochar bolus decreased the proportion of 2-mehtylbutryarte compared to BC, and when CO was added to BCB 2-methylbutyrate mol/ 100 mol increased. Similarly, with 1% and 4% inclusion of BC or BCB, 2-methylbuterate decreased as a proportion of total VFA. These decreases may be due to increased utilization of 2-methylbuterate for microbial growth. 2-methylbuterate is utilized for isoleucine synthesis in *Prevotella ruminicola* and by bacteria that cannot catabolize BCAA and must synthesize BCAA utilizing BCVFA (Robinson & Allision, 1969). *Ruminococcus flavefaciens* also incorporated labeled CO_2 with isobutyrate, isovalerate, and 2-methylbutyrate into valine, leucine, and leucine, respectively, which documented reductive carboxylation (Allison & Bryant, 1963). Other strains that require BCVFA for growth of major bacteria groups in the rumen include *Ruminococcus flavefaciens* strain C-94, *Ruminococcus flavefaciens* strain C1a, *Ruminococcus flavefaciens* strain B34b, *Ruminococcus albus* strain 7, and *Fibrobacter succinogenes* (Dehority et al., 1967). For valerate, there were interactions between fat supplementation and percent inclusion. When no fat was supplemented, 1% inclusion did not change; however, 2% and 4% inclusion of BC or BCB decreased valerate mol/100 mol. There was also a decrease in valerate mol/100 mol when fat was supplemented

with 1% and 2% inclusion of BC and BCB. This coincides with acetate molar proportion at 1% inclusion of BC and BCB with fat supplementation. At the 1% inclusion dose, acetate increased, but at 2% inclusion, acetate decreased which behaved the same as valerate with 2% inclusion. This decrease in valerate could be due to its utilization for growth in microbes such as *Fibrobacter succinogenes*, which requires valerate for growth and is used mainly in odd chain fatty acids and aldehydes (Wegner & Foster, 1963). In *Selenomonas ruminantium*, when bacteria were incubated with ^{14}C-valerate odd chain fatty acids were synthesized (Kanegasaki & Takahashi, 1967). When unlabeled, saturated fatty acids utilized valerate for fatty acid synthesis and it decreased. This shows the bacteria can utilize exogenous fatty acids for their membrane structure when provided with dietary fat instead of elongating valerate. This may partially explain why CO increased valerate mol/ 100 mol was added with BCB and why the effects of BCB or BC inclusions were not consistent in the two diets.

As a proportion of total VFA, caproate decreased with 1% or 4% inclusion of BC or BCB, and CO supplementation caused caproate mol/100 mol to increase. This increase with CO supplementation could result from bacteria utilizing the exogenous fatty acids instead of elongating more VFA for microbial membrane structure. Kanegasaki & Takahashi (1967) also showed that *S. ruminantium* incubated with ^{14}C-caproate synthesized even chain fatty acids. Like valerate when unlabeled saturated fatty acids were added, *S. ruminantium* utilized caproate less for fatty acid synthesis. This could explain why supplemental CO increased the molar proportion of caproate as less was used for microbial membranes.

Although methane gas production was not significant, there was numerical reduction of 23.08 mg produced in 24 hours ($P = 0.16$). This reduction of methane is consistent with an experiment that showed a numerical decrease of 11% and 17% for methane output across three samples of biochar when compared to the control (Hansen et al., 2012). Similarly, an experiment by Cabeza et al. (2018) demonstrated that the inclusion of biochar reduced total gas production to 0.96 ($P < 0.001$) and methane (CH_4) production to 0.95 ($P < 0.001$) when compared to the control. That experiment went on to test sources of biomass used for the biochar product and did not find changes in CH_4 production between sources (Cabeza et al., 2018). This study went on to analyze methane in grams per kg of NDF disappearance and found that methane decreased ($P = 0.022$) by 17.21 g/kg NDFD. As a result of not many experiments with biochar, this new comparison is important because it compared NDFD with methane, which are both important measurements for the implementation of biochar as a supplement. In addition to decreases in methane, a numerical decrease ($P = 0.23$) of 0.10 mg/d was also seen in hydrogen gas production. More studies on methane and hydrogen gas production will need to be conducted in anaerobic environments and *in vivo* models in order to fully evaluate biochar's potential to act as a methane mitigation strategy and the effects that it would have on production.

Conclusion:

In conclusion, biochar could reduce methane output without depressing NDFD and VFA when implemented as a feed additive. With the current stress on agricultural practices to decrease environmental impacts, feeding biochar as a methane mitigation strategy could be crucial to the dairy industry while simultaneously utilizing a waste product.

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