# Dose response of biochar and wood vinegar on in vitro batch culture ruminal fermentation using contrasting feed substrates

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**ABSTRACT:** Within Australia, approximately 6.4% of total greenhouse gas emissions are from animal methane ($CH_4$) derived from enteric fermentation. Mitigation of ruminant $CH_4$ is a key concept in support of sustainable agriculture production; dietary manipulations a viable strategy to lower $CH_4$ release during enteric fermentation. In order to determine the effects of dose response of biochar and wood vinegar supplementation on fermentation parameters and $CH_4$ production, this study utilized in vitro batch culture incubations. It is hypothesized that the addition of either biochar or wood vinegar will successfully reduce enteric $CH_4$ emissions without negative modification of other fermentation parameters. Three feed substrates (vegetable mixed ration, maize silage, and winter pasture) were separated into treatments containing either biochar at 0%, 0.5%, 1%, 2%, and 4% DM replacing substrate (w/w basis), or wood vinegar at 0%, 0.25%, 0.5%, 1%, and 2% into incubation media volume (v/v). At 6, 12, and 24 hours after inoculation, total gas volume, and methane ($CH_4$ %) were measured. Volatile fatty acid (VFA) concentrations, media pH, and in vitro dry matter digestibility were measured at 24 hours. Biochar at various dosages had no effect ($P > 0.05$) on fermentation characteristics other than decreased in vitro dry matter digestibility (IVDMD; $P = 0.01$) at 2% and 4% (DM basis) inclusion. Similar to biochar, dose response of wood vinegar had no effect on in vitro fermentation characteristics. However, feed substrate had major effects on all fermentation parameters ($P = 0.01$) where winter pasture > vegetable mixed ration > maize silage for all recorded fermentation characteristics. Biochar and wood vinegar supplementation were ineffectual in mitigating $CH_4$ production or modifying fermentation characteristics, thus rejecting the initial hypothesis. These results suggest the use of biochar is not an effective tool for methane mitigation in ruminant livestock and infers that studies previously reporting success must better define the systemic mechanisms responsible for the reduction in $CH_4$.

**Key words:** $CH_4$, dry matter digestibility, mitigation, volatile fatty acids



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## INTRODUCTION

Greenhouse gas (GHG) emissions present a global-scale challenge to sustainability. With 25 times the global warming potential of carbon dioxide (Houghton, 2001; Opio et al., 2013),

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methane extenuation strategies will be instrumental in supporting sustainable agriculture, particularly in ruminants.

Dietary amendment strategies intending to manipulate the rumen to mitigate $CH_4$ production have been long standing (Haque, 2018). Biochar, a by-product of biomass pyrolysis, has been identified by Meat and Livestock Australia as a methane mitigation research priority within their National Livestock Methane Program II (Cotter et al., 2015). The thermal conversion of biomass in an anaerobic enclosure forms a highly porous and inorganic carbonaceous residue, known as biochar (Leng et al., 2013). Its large surface area is theorized to promote formation of microbial biofilms in the rumen and absorb unwanted gases, as proposed by Leng et al. (2012) (Lehmann and Joseph, 2009; Leng et al., 2013; Saleem et al., 2018). Use of biochar as an additive to improve composting efficiencies has seen substantial success in a range of settings from pig manure to kitchen waste, with reported benefits for soil fertility due to its gas sequestration capabilities (Lehmann et al., 2008; Chowdhury et al., 2014; Yang et al., 2015; Joseph et al., 2015; Yu et al., 2020). This has extended to the application of biochar as an inorganic additive to cattle feed. Activated charcoal has been used for some time to prevent the absorption of toxicants (Toboika et al., 1995), and even combined with wood vinegar to treat calves for cryptosporidiosis (Watarai et al., 2008). There is significant debate over the successful and unsuccessful applications of biochar to reduce methane production in ruminants (Leng et al., 2012; Saleem et al., 2018; Cabeza et al., 2018; Winders et al., 2019; Teoh et al., 2019) While Leng et al. (2012, 2013) have previously demonstrated the effects of biochar on $CH_4$ production in ruminants, in-depth review of these articles is compromised by the differing processing methodologies and starting materials used, as well as variances in dosages and feed substrates. Further, this study specifically reports on the effects of dose, makes use of frequent sampling intervals, and compares a collection of substrates to provide a microanalysis of the proposed methane-reducing capabilities of biochar and novel use of wood vinegar, intending to lend insight as to the supplement's mode of action.

Previously, wood vinegar (WV), or pyroligneous acid, has primarily been used in environmental remediation. However, applications of WV are more expansive than even biochar, ranging from antifungal, termiticidal, and insect repellents to soil fertilizer (Takahara et al., 1993; Kiarie-Makara et al., 2010; Oramahi and Yoshimura, 2013). More recently, this by-product of wood carbonization has been investigated for its fermentative properties (Hua et al., 2020) and use in poultry and pig industries to improve growth performance and lean meat yield (Choi et al., 2009; Allahdo et al., 2017). Provided to chickens at 1% and 2% in drinking water, wood vinegar reportedly promotes lean meat production by regulation of lipid metabolism (Allahdo et al., 2017). Further, supplementing a weanling pig's basal diet with 0.2% WV has been found to significantly increase nutrient digestibility (Choi et al., 2009). This is thought to be due to its high proportion of organic acids that are readily absorbed for energy production (Choi et al., 2009). Second to water, wood vinegar's largest constituent is acetic acid, a final volatile fatty acid (VFA) and by-product of microbial digestion within the rumen. Although not previously used as a supplement for ruminants, it seems possible that the higher concentration of acetic acid will either disturb the rumen pH to suppress methanogenesis (Kessel and Russell, 1996; Zheng et al., 2017) or improve energy production as seen in pigs and poultry (Choi et al., 2009; Allahdo et al., 2017).

The study's primary hypothesis states that the addition of biochar at various doses will successfully reduce enteric $CH_4$ emissions without negative modification of other fermentation parameters when supplemented to multiple feed substrates. As a subsidiary hypothesis, it is theorized that the second by-product of biomass pyrolysis, wood vinegar, will have a similar effect, reducing methane production during enteric fermentation. As such, this study set out to test biochar supplementation replacing vegetable mixed ration, high-quality winter pasture and maize silage substrates, and wood vinegar supplemented on the concentration of media used during in vitro batch culture incubations, to determine the optimum supplementation dosage at which $CH_4$ production is minimized. As it is well known that diet composition has substantial influence over rumen microflora, and therefore fermentation kinetics (de Menezes et al., 2011), three substate feeds were used to determine if biochar or wood vinegar supplementation was more effective at reducing $CH_4$ production in one over another. In vitro dry matter digestibility (IVDMD), total gas (mL/g DM) and $CH_4$ production (mg $CH_4$/g DM), and VFA concentrations were measured to compare fermentative kinetics between the control and dosage treatments.

## MATERIALS AND METHODS

### *Experiment Design*

Three 24-hour in vitro batch-culture incubations were performed according to the method described by Terry et al. (2018), using a completely randomized design (CRD). Three feed substrates (vegetable mixed ration, maize silage, and winter pasture) were tested containing either biochar (0%, 0.5%, 1%, 2%, and 4%, biochar replacing substrate on w/w basis) or wood vinegar (0%, 0.25%, 0.5%, 1%, and 2%, v/v; wood vinegar to incubation media volume). Each treatment was incubated in triplicate giving a total of 93 incubation bottles on each incubation run ($n$ = 3) including blanks (e.g., three bottles with media only). Across 5 days, a total of three incubation runs of the above were carried out. The in vitro incubations were designed to measure total gas volume, $CH_4$ and VFA concentrations, pH change, and in vitro DM digestibility (IVDMD). The two treatment groups were tested separately and underwent individual statistical analysis.

### *Biochar and Wood Vinegar*

Biochar was obtained through slow pyrolysis (500 °C) of a combination of mixed species of green waste tree pruning. Wood vinegar (pH 3.1 measured at 21 °C) was generated from the combustion of fresh wood burning in anaerobic conditions. Both biochar and wood vinegar were provided from Cyclic Carbon Pty Ltd, an Australian company that forms carbon products from waste biomass. To ensure that particle size was small enough for inclusion in the experiment, the biochar used in the assay was the fraction that passed through a 1.0 mm screen. Chemical composition and physical properties of biochar (Rayment and Lyons, 2011) and volatile fatty acids profile of wood vinegar used are provided in Tables 1 and 2, respectively.

### *Substrate Feeds*

Three contrast feeds were used as substrates in the experiment: (1) vegetable mixed ration, (2) maize silage, and (3) winter pasture. The vegetable mixed ration was obtained from Kalfresh Cattle (Kalbar, QLD) on 24 January 2018. Random sampling of the vegetable mixed ration occurred from different locations once delivered to the trough by the mixer wagon (approximately 4 kg). The vegetable mixed ration contained approximately 60% carrots and pumpkin, 5% grass hay, 30% maize silage, 1.5% cottonseed meal, 1% vegetable oil, 1% sodium bentonite, and 1.5% commercial concentrate (as-fed basis). The other two substrate ingredients were obtained from Corstorphine Dairy (Cobbitty, NSW). An estimated 5 kg

**Table 1.** Chemical composition and physical properties of biochar

| Parameter | | Property | Parameter | | Property |
|---|---|---|---|---|---|
| Phosphorous (mg/kg P) | | 56 | Exchangeable Hydrogen | ($cmol_+$/kg) | <0.01 |
| | | | | (kg/ha) | <1 |
| | | | | (mg/kg) | <1 |
| pH | | 8.40 | Effective Cation Exchange Capacity (ECEC) ($cmol_+$/kg) | | 27 |
| Electrical conductivity | (dS/m) | 0.776 | Calcium (%) | | 60 |
| Exchangeable Calcium | ($cmol_+$/kg) | 16 | Magnesium (%) | | 8.5 |
| | (kg/ha) | 7,188 | | | |
| | (mg/kg) | 3,209 | | | |
| Exchangeable Magnesium | ($cmol_+$/kg) | 2.3 | Potassium (%) | | 20 |
| | (kg/ha) | 621 | Sodium – ESP (%) | | 12 |
| | (mg/kg) | 277 | Aluminium (%) | | 0.07 |
| Exchangeable Potassium | ($cmol_+$/kg) | 5.4 | Hydrogen (%) | | 0.00 |
| | (kg/ha) | 4,696 | Calcium/Magnesium Ratio | | 7.0 |
| | (mg/kg) | 2,097 | Total Carbon (%) | | 58 |
| Exchangeable Sodium | ($cmol_+$/kg) | 3.1 | Total Nitrogen (%) | | 0.52 |
| | (kg/ha) | 1,067 | Carbon/Nitrogen Ratio | | 112 |
| | (mg/kg) | 717 | Basic Texture | | Loam |
| Exchangeable Aluminium | ($cmol_+$/kg) | 0.02 | Basic Colour | | Black |
| | (kg/ha) | 3.5 | Chloride Estimate (equiv. mg/kg) | | 496 |
| | (mg/kg) | 1.6 | | | |

**Table 2.** Volatile fatty acid (VFA) profile of the wood vinegar used in incubations

| | Acetate | Propionate | Butyrate | BCVFA | Valerate | Caproate | Total VFA |
|---|---|---|---|---|---|---|---|
| Wood vinegar, mM | 3.51 | 1.46 | 0.91 | 0.62 | 0.10 | 0.06 | 6.66 |
| Wood vinegar, % of total VFA | 52.6 | 22.0 | 13.6 | 9.31 | 1.45 | 0.95 | |

BCVFA, branched-chain VFA (iso-butyrate + iso-valerate).

**Table 3.** Chemical composition of substrate feeds

| | Vegetable mixed ration diet | Maize silage | Winter pasture |
|---|---|---|---|
| | | % of dry matter | |
| Crude protein (CP) | 6.1 | 5.4 | 27.4 |
| Neutral detergent fiber (NDF) | 32.3 | 41.1 | 39.2 |
| Ether extract (EE) | 1.6 | 3.5 | 3.8 |
| Non-fibrous carbohydrates (NFC) | 44.1 | 45.5 | 22.4 |
| Ash | 15.9 | 4.5 | 7.2 |

NFC = 100 – (CP + NDF + EE + Ash).

wet winter pasture (Annual Ryegrass, *Lolium multiflorum L.*) was collected on 12 July 2017 at the Corstorphine Farm research site of the University of Sydney, Camden Campus, NSW, Australia (34° 04′ S; 150°81 69′E). The climate was warm-temperate with a mean annual minimum and maximum temperature of 10.7 and 23.3°C, respectively, and an annual rainfall of 738 mm (1900–2012). Various pasture samples were randomly selected and harvested at a grazing height of ≥5 cm above ground level to mimic grazing by cattle. Approximately 5 kg maize silage was randomly selected from different locations across the silage pit face. Dietary components were processed immediately after return to the laboratory. All substrate feeds were dried in an oven at 55 °C, ground and then passed through a 1 mm sieve using a feed mill (Model: Cutting Mill SM100, Retsch, Haan, Germany). Table 3 provides the chemical composition of the three substrates used in the experiment. The following AOAC (1995) methods were used to quantify dry matter (DM; Method 967.03), ash (Method 923.03), and ether extract (EE) content by extraction with petroleum ether using an Ankom Fat Extractor (Ankom Technol. Corp., Fairport, NY). Neutral detergent fiber (NDF) content was tested using sodium sulfite ($Na_2SO_3$) and amylase, adapted for the Ankom 200/220 Fiber Analyzer (Ankom Technol. Corp., Fairport, NY) and not corrected for ash or protein. Nitrogen (N) determination was assessed by the combustion method (Method # 990.03). Crude protein (CP) content, calculated as $N \times 6.25$, was applied for the nitrogen concentration determination.

### *Rumen Contents*

Rumen fluid was collected from two Droughtmaster rumen-cannulated steers. The steers were maintained at the University of Queensland, Gatton and were taken care of in accordance with the guidelines of the Animal Ethics Committee (Approval protocol number: AE35581). The steers were fed at maintenance level on a pasture diet with *ad libitum* access to water prior to rumen fluid collection, which was extracted within 2 hours after morning feeding. The pooled rumen fluid was immediately filtered through a 4-layer cheesecloth into a pre-warmed and insulated thermos and utilized as inoculum within 15 min of collection.

### *Batch Culture In Vitro Incubation*

All substrate feeds and biochar were proportionally weighed (500 ± 50 mg) into F57 Ankom bags (Ankom Technology) with three replicates per treatment and sealed. On the day of incubation, each bag was placed into a 50 mL amber serum bottle ($n$ = 3) and wood vinegar was pipetted into the bottles accordingly with its corresponding concentration (0, 0.0625, 0.125, 0.25, and 0.5 mL). The bottles were warmed to 39 ± 0.5 °C in an incubator for 60 min and gassed with $CO_2$ to remove oxygen. Incubation media (25 mL), consisting of rumen fluid and buffer (1:2 ratio) (Terry et al., 2018), was added to the bottles, which were then sealed with rubber stoppers and incubated for 24 hours at 39 ± 0.5 °C. This incubation procedure was repeated three times with three replicates (e.g., serum bottles) for each treatment.

### *Determination of $CH_4$ Concentration, Total Gas Production, and pH*

Methane samples were obtained by removing each bottle at the allocated sampling time (6, 12, and 24 hours) and collecting 17 mL of headspace gas through insertion of a 25 mL syringe through the septum bottle. Sampled gas was then injected into a 10 mL evacuated exetainer where a 3 mL sub-sample was transferred into an evacuated head-space vial and then analyzed for $CH_4$ concentration using gas chromatography (GC) (Agilent 7890A, Agilent Technologies, Santa Clara, CA). The GC was installed with a capillary column (Restek Rt-Q-Bond, 30 m × 0.53 mm ID × 20 µm) and was equipped with a flame ionization detector (FID; air flow 300 mL/min, $H_2$ fuel flow 30 mL/min, and makeup flow ($N_2$) 30 mL/min) at 250 °C. Injector splitless type (9.526 PSI, Helium total flow 33 mL/min, septum purge flow 3 mL/min, and split flow 25 mL/min) used in split mode to 50: 1 at 60 °C. Oven temperature was maintained at 60 °C. The calibration curves were performed with reference standards for $CH_4$ concentrations, as follows: 0%, 4.82%, 10.72%, and 20%. Amounts of $CH_4$ were then calculated and presented as both percentage and mg/g DM.

Total gas production was measured at 6, 12, and 24 hours of incubation. Water displacement apparatus (Fedorah and Hrudey, 1983) was applied to determine total gas production after the gas sample for $CH_4$ was taken. Total gas was then calculated by adding the gas measured from water displacement apparatus to the amount of gas sample taken for $CH_4$ determination.

Media pH was measured at the end of the 24 hours incubation by using a pH meter (Model WP-80, pH-mV-Temp. Meter, TPS Pty Ltd, Brisbane, Australia) calibrated at 39 °C. The incubation bottles were then placed on ice to terminate fermentation.

### *Determination of Volatile Fatty Acids Concentrations and In Vitro DM Digestibility*

Volatile fatty acid concentrations were analyzed using GC (Agilent 7820A, Agilent Technology, Santa Clara, CA). The GC was equipped with an Agilent column (30 m × 0.32 mm × 1.00 µm) and flame ionization detector (FID; $N_2$ make-up 25–30 mL/min; $H_2$, 30 mL/min; air 300 mL/min). The temperature was 225 °C in the injector and 250 °C in the FID. Helium (velocity of 28.5 cm/s) was the carrier gas and the injector split type was used in split mode to 50:1. Individual samples (1.5 mL) from each bottle was transferred into a 2 mL centrifuge microtube and then de-proteinized by adding 0.3 mL metaphosphoric acid (0.25; w/v). These samples were stored at −20 °C between collection and analysis. Samples were thawed overnight at 4 °C and centrifuged at 12,000 rpm for 2 min. After that, 1.2 mL of the supernatant in each microtube were transferred into a 2 mL centrifuge tube and 0.2 mL crotonic acid were added as an internal standard (Playne, 1985). After centrifugation for 10 min at 12,000 rpm, 1.1 mL supernatant was transferred to a 2 mL autosampler vial and transferred onto the GC tray for analysis. Three quality controls (low, medium, and high VFA) were prepared and tested after every 20 samples. Each standard curve solution (acetic, propionic, and butyric) was also prepared and analyzed with following acid mM concentrations: 0, 5, 8, 10, 25, 50, 75, and 100 mM. Other VFA standard curves (iso-butyric, butyric, iso-valeric, valeric, and caproic) were also tested but with different acid mM concentrations (0.0, 0.5, 0.8, 1.0, 2.5, 5.0, 7.5, 10.0 mM). The amount of sample VFA (mM) was then calculated by the standard curves using area ratio. Total VFA concentration and percentages of individual VFA were finally calculated and presented as mM and % of total VFA, respectively. The VFA samples from in vitro incubation run 1 were neither GC analyzed nor included in the statistical analysis because of power failure in the freezer during storage.

Ankom® bags with residues were removed from the bottles after incubation and washed with distilled water using the Ankom Fiber Analyzer (2 cycles of 10 min at 100 °C), oven-dried at 55 °C for 48 hours and weighed to estimate IVDMD.

### *Statistical Analysis*

In vitro incubations were repeated three times in three complete runs, with three replicates per treatment in each run. The three replicates were averaged before statistical analysis and those averages, within runs, contributed to the statistical unit. The data was analyzed using the MIXED procedure of SAS (2020) by sampling time. Biochar and wood vinegar were analyzed separately as completely randomized design as dose, substrate feed, and dose × substrate feed as a fixed effect, and the run within treatment interaction as random effects. Parameters with a

significant dose × substrate feed interaction will be sliced by substrate feed. The run by treatment interaction was used as the error to test the treatment effect. Differences among means were tested using the least square linear hypothesis test with the level of significance at $P < 0.05$.

## RESULTS

### *Effect of Biochar on Dry Matter Digestibility, VFA Concentrations, pH, Total Gas, and Methane Production*

There were minor effects of the addition of biochar on fermentation characteristics. There was a decrease ($P < 0.01$) in IVDMD at 2 and 4% of biochar replacing substrate compared to the control treatment. Interaction dose × substrate feed was significant for pH and Figure 1A slices the interaction and illustrated differences in maize silage and winter pasture only, reporting pH increase at 0.5% biochar supplementation ($P < 0.05$; Figure 1A) compared to the control maize substrate. Winter pasture had lower ($P < 0.05$) pH at 1% supplementation compared to its respective control. The remaining parameters were presented separately for dose and substrate feed (Table 4), whereby the addition of biochar had no effect on total gas (mL/g DM) ($P \geq 0.09$), $CH_4$ as a % of total gas ($P \geq 0.53$) or $CH_4$ production (mg $CH_4$/g DM) ($P \geq 0.54$). Feed substrate affected all fermentation characteristics ($P < 0.01$; Table 4). Overall, fermentation parameters were greater in winter pasture than

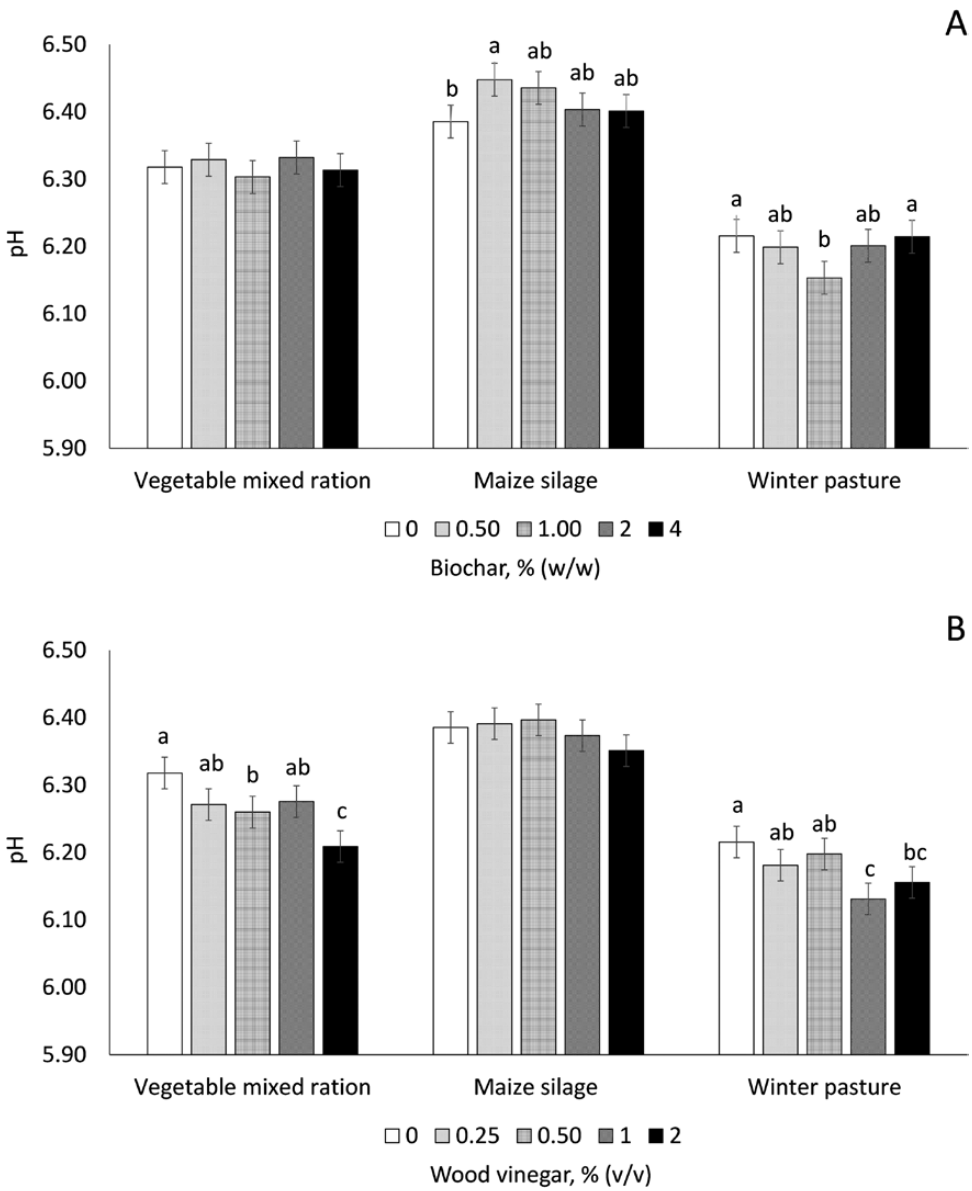


**Figure 1.** Interaction dose × substrate feed sliced by substrate feed for media pH at 24 hours at increasing doses of biochar (A) and wood vinegar (B). Means values within substrate feed with different letters differ ($P < 0.05$).

**Table 4.** Effects of increasing concentrations of biochar replacing substrates (vegetable mixed ration (veg), maize silage, and winter pasture) on in vitro fermentation characterization at different sampling times

| | | Biochar (w/w) | | | | | | Substrate feed (SF) | | | | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0% | 0.50% | 1% | 2% | 4% | SEM dose | Veg | Maize silage | Winter pasture | SEM SF | Dose | SF | Dose × SF |
| Total gas, mL/g DM | 6 h | 37.3 | 37.3 | 38.4 | 38.9 | 40.7 | 2.95 | 43.5$^{b}$ | 20.8$^{c}$ | 51.2$^{a}$ | 2.81 | 0.34 | <0.01 | 0.16 |
| | 12 h | 74.3 | 73.0 | 74.6 | 75.6 | 77.5 | 3.18 | 81.1$^{b}$ | 52.9$^{c}$ | 90.9$^{a}$ | 3.10 | 0.09 | <0.01 | 0.14 |
| | 24 h | 111.6 | 111.7 | 113.3 | 115.9 | 116.9 | 4.48 | 118.2$^{b}$ | 95.3$^{c}$ | 128.1$^{a}$ | 4.31 | 0.18 | <0.01 | 0.29 |
| CH4, % | 6 h | 4.32 | 4.24 | 4.67 | 4.75 | 4.56 | 0.535 | 5.36$^{a}$ | 2.94$^{b}$ | 5.23$^{a}$ | 0.511 | 0.53 | <0.01 | 0.38 |
| | 12 h | 7.13 | 6.51 | 6.75 | 6.58 | 7.13 | 0.843 | 6.94$^{a}$ | 6.00$^{b}$ | 7.52$^{a}$ | 0.807 | 0.66 | <0.01 | 0.88 |
| | 24 h | 6.45 | 7.05 | 6.99 | 6.79 | 6.43 | 0.541 | 7.60$^{a}$ | 5.95$^{b}$ | 6.67$^{b}$ | 0.485 | 0.66 | <0.01 | 0.11 |
| mg CH4/g DM | 6 h | 0.63 | 0.62 | 0.70 | 0.72 | 0.69 | 0.114 | 0.83$^{b}$ | 0.22$^{c}$ | 0.97$^{a}$ | 0.110 | 0.54 | <0.01 | 0.51 |
| | 12 h | 1.58 | 1.46 | 1.59 | 1.56 | 1.60 | 0.219 | 1.75$^{b}$ | 0.90$^{c}$ | 2.01$^{a}$ | 0.213 | 0.74 | <0.01 | 0.96 |
| | 24 h | 2.46 | 2.42 | 2.55 | 2.53 | 2.53 | 0.209 | 2.75$^{a}$ | 1.82$^{b}$ | 2.93$^{a}$ | 0.198 | 0.90 | <0.01 | 0.49 |
| IVDMD | 24 h | 63.9$^{a}$ | 63.4$^{ab}$ | 64.7$^{a}$ | 62.3$^{bc}$ | 61.4$^{c}$ | 0.68 | 64.1$^{b}$ | 53.7$^{c}$ | 71.7$^{a}$ | 0.62 | <0.01 | <0.01 | 0.22 |
| pH | 24 h | 6.31 | 6.33 | 6.30 | 6.31 | 6.31 | 0.020 | 6.32 | 6.41 | 6.20 | 0.018 | 0.61 | <0.01 | 0.05 |

SEM, standard error of the means; IVDMD, in vitro dry matter digestibility; DM, dry matter.
Within single fixed effect (biochar or diet), means values with different letters differ ($P < 0.05$).

vegetable mixed ration diet, and maize silage, respectively (Table 4).

There were no effects of biochar dose on total VFA concentration (mM) ($P = 0.77$; Table 5) or percentages of individual VFA of total VFA ($P > 0.33$; Table 5). Feed substrate had a major effect on total VFA concentrations (mM), percentages of individual VFA of total VFA and acetate to propionate ratio (Table 5). Total VFA concentration was greater ($P < 0.01$) for winter pasture than vegetable mixed ration, also greater than maize silage. BCVFA (% of total VFA) and valerate (% of total VFA) were greater ($P < 0.01$) in maize silage than the vegetable ration diet and winter pasture, while butyrate (% of total VFA) was greater in the vegetable ration than maize silage and winter pasture. Propionate (% of total VFA) was greater ($P <0.01$) in maize silage > winter pasture > vegetable, respectively. While A:P ratio showed the reverse order. There were no dose × substrate interactions recorded for VFA, but results did show tendency for increased acetate (% of total VFA) when biochar was included ($P = 0.07$).

### *Effect of Wood Vinegar on Dry Matter Digestibility, VFA Concentrations, pH, Total Gas, and Methane Production*

Out of all the fermentation characteristics, wood vinegar supplementation had an effect on pH only (Table 6). Similar to the biochar results, interaction dose × substrate feed was significant for pH as illustrated in Figure 1B. Wood vinegar supplementation had no influence on pH for the maize silage substrate, irrespective of dosage ($P > 0.05$; Figure 1B). Media pH was reduced at 1% and 2% wood vinegar supplementation for winter pasture but did not differ between the 1% and 2% dosages ($P < 0.05$; Figure 1B). For the vegetable mixed ration diet, pH decreased at 0.5% compared to the control, however the reduction was greatest at 2% wood vinegar dosage ($P < 0.05$; Figure 1B). Further, substrate had a major effect on all fermentation characteristics (Table 6), where total gas (mL/g DM) and IVDMD were greater ($P < 0.01$) for winter pasture than vegetable mixed ration diet, and maize silage at 24 hours. $CH_4$ (%) was greater ($P < 0.01$) in vegetable mixed ration compared to winter pasture and maize silage at 24 hours. For $CH_4$ production (mg $CH_4$/g DM) the vegetable mixed ration diet was equal to winter pasture but greater than maize silage at 24 hours.

An interaction between dose × substrate feed for BCVFA (% of total VFA) was observed ($P = 0.03$;

**Table 5.** Total volatile fatty acid (VFA) concentration (mM), percentages of individual VFA of total VFA and acetate to propionate ratio from in vitro fermentation by the addition of biochar in vegetable mixed ration (veg), maize silage, and winter pasture

| | Biochar (w/w) | | | | | | Substrate feed (SF) | | | | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0% | 0.50% | 1% | 2% | 4% | SEM dose | Veg | Maize silage | Winter pasture | SEM SF | Dose | SF | Dose × SF |
| Total VFA (mM) | 114.9 | 110.9 | 112.0 | 114.0 | 113.5 | 4.60 | 117.5$^{b}$ | 100.0$^{c}$ | 121.7$^{a}$ | 4.14 | 0.77 | <0.01 | 0.54 |
| Percentages of individual volatile fatty acids of total VFA | | | | | | | | | | | | | |
| Acetate (A) | 59.2 | 59.6 | 59.7 | 59.7 | 59.8 | 0.95 | 60.2$^{a}$ | 58.7$^{b}$ | 59.9$^{a}$ | 0.94 | 0.33 | <0.01 | 0.07 |
| Propionate (P) | 26.0 | 25.9 | 25.8 | 25.9 | 25.7 | 0.80 | 25.3$^{c}$ | 26.4$^{a}$ | 25.9$^{b}$ | 0.79 | 0.75 | <0.01 | 0.65 |
| Butyrate | 11.0 | 10.8 | 10.8 | 10.7 | 10.9 | 0.31 | 11.1$^{a}$ | 10.8$^{b}$ | 10.7$^{b}$ | 0.31 | 0.37 | <0.01 | 0.82 |
| BCVFA | 2.29 | 2.21 | 2.12 | 2.21 | 2.15 | 0.272 | 1.96$^{b}$ | 2.52$^{a}$ | 2.10$^{b}$ | 0.256 | 0.84 | <0.01 | 0.27 |
| Valerate | 1.18 | 1.18 | 1.17 | 1.16 | 1.17 | 0.062 | 1.10$^{b}$ | 1.33$^{a}$ | 1.08$^{b}$ | 0.061 | 0.92 | <0.01 | 0.97 |
| A:P ratio | 2.27 | 2.30 | 2.31 | 2.31 | 2.33 | 0.106 | 2.38$^{a}$ | 2.22$^{c}$ | 2.31$^{b}$ | 0.105 | 0.56 | <0.01 | 0.39 |

SEM, standard error of the means; BCVFA, branched-chain VFA (iso-butyrate + iso-valerate).

Within fixed effect of diet, means values with different letters differ ($P < 0.05$).

**Table 6.** Effects of increasing concentrations of wood vinegar replacing substrates (vegetable mixed ration (veg), maize silage, and winter pasture) on in vitro fermentation characterization at different sampling times

| | | Wood vinegar (v/v) | | | | | | Substrate feed (SF) | | | | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0% | 0.25% | 0.50% | 1% | 2% | SEM dose | Veg | Maize silage | Winter pasture | SEM SF | Dose | SF | Dose × SF |
| Total gas, mL/g DM | 6 h | 37.3 | 40.6 | 40.5 | 40.5 | 41.4 | 2.43 | 46.1$^{a}$ | 22.7$^{b}$ | 51.4$^{c}$ | 2.24 | 0.32 | <0.01 | 0.35 |
| | 12 h | 74.1 | 76.9 | 76.9 | 76.0 | 77.1 | 2.81 | 83.9$^{b}$ | 54.5$^{c}$ | 90.2$^{a}$ | 2.61 | 0.62 | <0.01 | 0.13 |
| | 24 h | 111.6 | 117.2 | 116.6 | 115.1 | 116.9 | 3.03 | 121.6$^{b}$ | 97.1$^{c}$ | 127.7$^{a}$ | 2.84 | 0.18 | <0.01 | 0.15 |
| CH4, % | 6 h | 4.32 | 4.60 | 4.47 | 4.12 | 5.13 | 0.776 | 5.01$^{a}$ | 3.41$^{b}$ | 5.17$^{a}$ | 0.750 | 0.22 | <0.01 | 0.16 |
| | 12 h | 7.13 | 7.64 | 6.89 | 6.64 | 7.56 | 0.817 | 7.03$^{ab}$ | 6.58$^{b}$ | 7.90$^{a}$ | 0.701 | 0.69 | 0.02 | 0.22 |
| | 24 h | 6.45 | 6.19 | 6.75 | 6.18 | 6.62 | 0.653 | 7.52$^{a}$ | 5.80$^{b}$ | 5.99$^{b}$ | 0.598 | 0.82 | <0.01 | 0.56 |
| mg CH4/g DM | 6 h | 0.63 | 0.70 | 0.73 | 0.63 | 0.79 | 0.817 | 0.83$^{b}$ | 0.29$^{c}$ | 0.97$^{a}$ | 0.135 | 0.24 | <0.01 | 0.54 |
| | 12 h | 1.58 | 1.70 | 1.63 | 1.49 | 1.76 | 0.239 | 1.79$^{b}$ | 1.05$^{c}$ | 2.06$^{a}$ | 0.227 | 0.42 | <0.01 | 0.39 |
| | 24 h | 2.46 | 2.60 | 2.60 | 2.36 | 2.71 | 0.224 | 2.81$^{a}$ | 1.97$^{b}$ | 2.87$^{a}$ | 0.213 | 0.22 | <0.01 | 0.25 |
| IVDMD | 24 h | 63.9 | 64.9 | 64.0 | 64.3 | 63.4 | 0.76 | 65.5$^{b}$ | 54.6$^{c}$ | 72.1$^{a}$ | 0.67 | 0.39 | <0.01 | 0.70 |
| pH | 24 h | 6.31 | 6.28 | 6.28 | 6.26 | 6.24 | 0.02 | 6.27 | 6.38 | 6.18 | 0.018 | 0.03 | <0.01 | 0.01 |

SEM, standard error of the means; IVDMD, in vitro dry matter digestibility; DM, dry matter.

Within single fixed effect (wood vinegar or diet), means values with different letters differ ($P < 0.05$).

Table 7) in only vegetable mixed ration diet, where the addition of wood vinegar at any concentration decreased BCVFA by 24% (% of total VFA) compared to the control. Dosages of wood vinegar had no effect on total VFA or individual VFA ($P \geq 0.15$). Similarly, with all previous results, substrate affected total VFA, percentages of individual VFA of total VFA and A:P ratio (Table 7). Total VFA and acetate (% of total VFA) were greater ($P < 0.01$) in winter pasture than maize silage but equal to vegetable mixed ration. Propionate (% of total VFA) was greater in maize silage than winter pasture > vegetable mixed ration. Butyrate (% of total VFA) was greater ($P < 0.01$) in the vegetable mixed ration than maize silage and winter pasture, while valerate was highest ($P < 0.01$) in maize silage > vegetable mixed ration > winter pasture, respectively. The vegetable mixed ration had the greatest A:P ratio, while maize silage had the lowest ($P < 0.01$, Table 7).

## DISCUSSION

Neither biochar nor wood vinegar supplementation successfully reduced $CH_4$ production or had beneficial effects on fermentation parameters, effectively denying the initial hypotheses. It has been well documented that biochar particle size, electrical conductivity, adsorptive potential, and ability to manipulate biofilms all differ with biomass source and processing methodologies (Hansen et al., 2012; Leng et al., 2013; Yu et al., 2015; McFarlane et al., 2017; Wang et al., 2018). Two recent publications suggest acidic carbon-rich biochar has a higher redox potential, and therefore electron-donating capacity, with potential to manipulate microbial populations (Cruz Viggi et al., 2017; Huang et al., 2018). Yet, consistency seems an obvious barrier to definitive confirmation. Two recent papers (Teoh et al., 2019; Terry et al., 2019) both used biochars with a pH similar to that applied in the current study (8.2, 7–8, and 8.4, respectively). All investigations reported no effect of the biochar on methane production. However, Winders et al. (2019) described a 9.6% reduction in $CH_4$ production with a biochar pH of 8.0, as did Saleem et al. (2018) with a biochar pH of 4.8 (25.2% reduction in $CH_4$). This suggests that although there may be a vague motion that biochar pH could have some effect on the degree of $CH_4$ reduction, major inter-study inconsistencies in biomass source and processing methodologies prevent the confirmation of pH as a determinative factor influencing the methane reducing potential of biochar.

**Table 7.** Total volatile fatty acid (VFA) concentration (mM), percentages of individual VFA of total VFA and acetate to propionate ratio from in vitro fermentation by the addition of wood vinegar in vegetable mixed ration (veg), maize silage, and winter pasture

| | Wood vinegar (v/v) | | | | | | Substrate feed (SF) | | | | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0% | 0.25% | 0.5% | 1% | 2% | SEM dose | Veg | Maize silage | Winter pasture | SEM SF | Dose | SF | Dose × SF |
| Total VFA (mM) | 114.9 | 114.6 | 116.8 | 117.4 | 115.4 | 2.71 | 121.8[a] | 103.7[b] | 123.2[a] | 1.89 | 0.83 | <0.01 | 0.07 |
| Percentages of individual volatile fatty acids of total VFA | | | | | | | | | | | | | |
| Acetate (A) | 59.2 | 59.4 | 59.5 | 59.4 | 59.7 | 0.91 | 60.1[a] | 58.5[b] | 59.7[a] | 0.89 | 0.79 | <0.01 | 0.48 |
| Propionate (P) | 26.0 | 26.1 | 26.1 | 26.1 | 25.8 | 0.66 | 25.3[c] | 26.6[a] | 26.1[b] | 0.63 | 0.87 | <0.01 | 0.97 |
| Butyrate | 11.0 | 10.9 | 10.8 | 10.8 | 10.8 | 0.35 | 11.3[a] | 10.6[b] | 10.7[b] | 0.34 | 0.68 | <0.01 | 0.54 |
| BCVFA | 2.29 | 2.15 | 2.13 | 2.24 | 2.19 | 0.183 | 1.83 | 2.65 | 2.12 | 0.167 | 0.77 | <0.01 | 0.03 |
| Valerate | 1.18 | 1.18 | 1.14 | 1.14 | 1.13 | 0.052 | 1.10[b] | 1.29[a] | 1.08[b] | 0.051 | 0.15 | <0.01 | 0.66 |
| A:P ratio | 2.27 | 2.28 | 2.28 | 2.27 | 2.32 | 0.092 | 2.38[a] | 2.20[c] | 2.29[b] | 0.089 | 0.82 | <0.01 | 0.79 |

SEM, standard error of the means; BCVFA, branched-chain VFA (iso-butyrate + iso-valerate).
Within fixed effect of substrate feed, means values with different letters differ ($P < 0.05$).

A study hypothesized that the effectivity of biochar is dependent on its potential to provide an improved location for biofilm microbial consortia, facilitating oxidation of $CH_4$ via methanotrophic organisms (Leng et al., 2013). This theory is largely based on the large surface area to weight ratio, opportunistically increasing microorganism attachment and absorption of gases (Leng et al., 2013). This may explain the success of Leng et al. (2012) who described a 22% reduction in $CH_4$ using rice husk biochar processed at 900 °C, while this study used biochar processed at 500 °C; higher temperatures are thought to influence sorption capacity (Lehmann and Joseph, 2009). Alternatively, Cabeza et al. (2018) reported no differences in $CH_4$ production between biomass sources (*Miscanthus* straw pellets, oil seed straw pellets, rice husks, soft wood pellets, and wheat straw pellets), but the greatest reduction in $CH_4$ production was seen in biochar processed at 550 °C rather than 700 °C (8.8–4.8% vs. 0.5–5.4% $CH_4$ reduction). These contrasting results suggests the $CH_4$ reduction activity of biochar during enteric fermentation may be unrelated to biochar surface area or porosity in a rumen fluid setting, unlike in a soil environment.

The results of this study negate a dose–response relationship between biochar and $CH_4$ production. As biochar is 100% inorganic, the biological feasibility of microorganisms attaching to the indigestible supplement seems unlikely when there are competing, already-established biofilms. Teoh et al. (2019) found no microbial attachment to hardwood biochar after 48 hours of fermentation in a RUSITEC system using scan electron microscopy (data not presented). Further, biochar supplementation concentrations required to alter $CH_4$ production are majorly inconsistent amongst studies. Saleem et al. (2018) added biochar at only 0.5% in the diet DM for a 25.2% reduction in $CH_4$ mg/d, yet Hansen et al. (2012) replaced 9% of feed DM with biochar for a numerical but not significant $CH_4$ reduction. Similarly, Teoh et al. (2019) reported a tendency ($P = 0.10$) for $CH_4$ (%) to decrease when increasing hardwood biochar dosage from 0 or 400 mg/d to 800 mg/d but $CH_4$ production (mg/d) was not altered by biochar supplementation. Yet, increasing dosage from 0.5% to 4% DM failed to produce resulting effects in the current study using in vitro incubations. These notable disparities limit the practical implementation of biochar additives, given its indigestible composition acting as a form of energy-dilution. This explains the associated reduction of IVDMD and total VFA as biochar dosage increases as it provides no nutrient value to the live animal while contributing considerably to rumen fill at high dosages.

Currently, speculation surrounds the systemic mechanisms responsible for the reduced $CH_4$ production seen in Leng et al. (2012) and Saleem et al. (2018). Yet, the findings of both this study and Teoh et al. (2019) suggest it is likely unrelated to the absorption of gases within biochar or establishment of alternative biofilms within the rumen.

This study failed to identify any significant change in enteric $CH_4$ production after wood vinegar supplementation up to 2% (v/v). Suresh et al. (2019) confirmed the antimicrobial efficacy of wood confirmed the antimicrobial efficacy of wood vinegar which may explain the 24% reduction (2.27% of BCVFA in total VFA at 0% WV upplementation vs. 1.72% of BCVFA in total VFA averaged across all other WV concentrations in veg diet) in % of BCVFA (iso-butyrate + iso-valerate) of total VFA in the vegetable mixed diet only, given their role in ruminal microbial growth, function, and enzyme activity (Andries et al., 1990; Moharrey, 2004). Although not as notable as expected given the predominant acetate constituent of the wood vinegar (52.6%), there was a tendency for acetate (% of total VFA) to increase alongside wood vinegar dosage. Yet, this did not cause a change in total VFA concentration, likely due to interconversion of acetic acid by the microbial populations (Hackmann and Firkins, 2015). The drop in pH seen in the vegetable ration diet is likely a result of the design of the in vitro system where VFA would usually be absorbed through the rumen wall to prevent such pH reductions and contribute to ATP production (Danielli et al., 1945; Qumar et al., 2016). Regardless, the identified pH change of 1.1% is unlikely to be biologically influential due to the small scale (Figure 1B).

Suresh et al. (2019) reports that the antibacterial properties of wood vinegar are greatest at a neutral pH (7.0), suggesting its inhibitory efficiencies can be attributed to the antimicrobial effect of ketones rather than the presence of acetic acid. Further, the presence of phenols and heterocyclic compounds in wood vinegar are known to have toxic effects on anaerobic microbial activity (Zheng et al. 2017). The high percentage of acetic acid (of the total VFA) within wood vinegar may compromise the practicability of the additive. Whether wood vinegar's unpalatability makes the supplement detrimental to overall feed intake is yet to be evaluated.

There was no difference in $CH_4$ production between substrate feeds with a biochar or wood vinegar additive, and those without. However, the differential composition of the feed substrates is

largely responsible for variations in fermentation parameters seen between diets in the current study (Cui et al., 2019; Li et al., 2019). It has been well documented that diet has considerable influence on the structure of rumen flora and its corresponding fermentation kinetics (de Menezes et al., 2011). Feed chemical compositions have identified nutritional indices with the greatest influence on ruminal fermentation; hemicellulose and cellulose as the main source of energy degraded into $CH_4$ via rumen microbes (Li et al., 2019). Similarly, crude protein is said to increase digestibility by supporting self-replication and enzyme synthesis of bacteria (Russell et al., 1983; Li et al., 2019), confirmed by winter pasture, seen to have the greatest CP and corresponding $CH_4$, % and mg $CH_4$/g DM. This suggests that while the chemical composition of substrates have major influence over total gas production and $CH_4$ concentration, it does not directly translate to control over the $CH_4$ mitigation potential of biochar or wood vinegar.

Current literature fails to describe the physiological effects of long-term biochar or wood vinegar supplementation; if there are environmental implications or pollution risks once they are excreted, or if the ability to suppress methanogenic pathways is longstanding. Further investigation and refinement of information is needed to determine the systemic mechanism of biochar that allows for an adjustment of fermentation kinetics, particularly if large corporations such as Meat and Livestock Australia are hoping to use biochar as an in-market livestock methane mitigation technology. With many questions surrounding the biomechanics of biochar largely unanswered by current literature, and few explanations provided by studies that have successfully reduced $CH_4$ production, there is very little reliable data available to draw justifiable conclusions. Given the recent attention given to biochar as a $CH_4$ mitigation tool, all findings, including this study, must be considered to fully appreciate the viability of biochar for use as animal feed.

The $CH_4$ mitigation potential of biochar and wood vinegar have not been demonstrated or were not able to be replicated in this study using contrast feed substrates. Queries remain as to whether inorganic additives can influence rumen fermentation due to their indigestible conformation or high variability in post-processing properties. The lack of standardized methodology during biochar production is majorly confounding, preventing comparative analysis of past literature. Consequently, the $CH_4$ mitigation potential of biochar and wood vinegar remains poorly defined.

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