Final report

Fit-for-purpose biochar to improve efficiency in ruminants

Project code: B.GBP.0032

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Date published: 04 April 2022

PUBLISHED BY
Meat & Livestock Australia Limited
PO Box 1961
NORTH SYDNEY NSW 2059

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Abstract

Biochar has attracted interest as an additive for ruminants because of its anti-methanogenic potential, although *in vivo* results are limited and sometimes contradictory. Our objectives were to manipulate the process of biochar production to produce fit-for-purpose biochar for ruminant production; use a range of different substrates during this process; and identify a dose that maximises productivity and minimises emissions from livestock systems, and which can be incorporated into a palatable livestock diet. Five *in vitro* experiments were completed and two biochars out of 14 candidates were selected and tested in cattle (three animal trials) to study their effects on enteric methane, rumen fermentation and animal productivity under controlled feeding conditions and extensive grazing. When tested *in vitro*, we observed a great variability on methane reduction, mainly driven by the biomass type used, pyrolysis temperature, pre- and post- pyrolysis manipulation and dose rate. The extent of the methane reduction *in vivo* (under controlled feeding conditions) was lower than the reduction observed *in vitro* (8.8-12.9% vs 23-33 %). However, the same biochars did not successfully decrease methane emissions or improve productivity under extensive grazing conditions. Further research will be required to identify a fit-for-purpose biochar suitable for grazing systems. The successful candidate would require a much greater methane reduction than we observed under controlled feeding conditions to be suitable to be used as anti-methanogenic supplement under grazing conditions.

Executive summary

Background

Over the last decade, enteric CH_4 production by livestock has been targeted by ruminant nutritionists because of its contribution to anthropogenic greenhouse gas emissions. In recent years, biochar has attracted interest as an additive for ruminants because of its anti-methanogenic potential, although *in vivo* results are limited and sometimes contradictory. Some producers are interested in feeding biochar to cattle and sheep because there is evidence that soil health is improved through distribution of biochar in the soil, with help from dung beetles. In this project the biochar process was manipulated in an attempt to produce fit-for-purpose biochar for ruminants to manage enteric methane production.

Objectives

- Manipulate the process of biochar production to produce fit-for-purpose biochar for ruminant production.
- Use a range of different substrates to help generate fit-for-purpose biochar.
- Identify a dose that maximises productivity and minimises emissions from livestock systems and that can be incorporated into a palatable diet for livestock.

The biochars tested were not able to decrease methane emissions or increase productivity when fed to cattle under extensive grazing conditions. Some biochars were able to decrease methane (up to 41%) *in vitro* and two of them inhibited enteric methane (8.8-12.9%) emissions when fed to animals under controlled feeding conditions with non-detrimental effect on rumen fermentation or animal feed intake.

Results/key findings

- The 14 biochars tested showed a wide variability on methane inhibition, from no effect on methane production to up to a 41% inhibition *in vitro*. Our findings confirmed that not all biochars are able to decrease enteric methane. The effect is dependent on different characteristics such as the biomass used, pyrolysis temperature, pre- and post- pyrolysis manipulation, dose rate and additional compounds in the biochar.
- Two biochar types – a custom-made biochar (Biochar 4.2 NT6) and a commercial biochar (Biochar 6) showed the strongest potential to inhibit methane in ruminants and were tested *in vivo*. Their anti-methanogenic effects were likely to be due to different properties: the custom-made biochar contained nitrates, which are an indirect inhibitor of methanogenesis, was acidic, based on a substrate mix of *Eucalyptus globulus*, straw, bentonite, zeolite and pyrolysis temperature of 600°C. In contrast, the commercial biochar was alkaline, based on the parent substrate *Acacia cambagei* and pyrolysis temperature of 450°C. The results suggest that the mode of action of the biochars tested is likely to be through indirect inhibition of methanogenesis.
- The two biochars and doses tested reduced CH_4 emissions (8.8-12.9 % reduction) in cattle under controlled feeding conditions without any detrimental effect on rumen fermentation or DMI.
- Under grazing conditions no significant difference on enteric CH_4 emissions or productivity were detected when the same biochars were supplemented to cattle over 60 d.

Future research and recommendations

The results of this project demonstrate that it is possible to manipulate biochars to have different effects on ruminal fermentation and methane production. Further research will be required to identify a fit-for-purpose biochar with much greater anti-methanogenic properties (3-4 times or more than current project) to be viable for Australian grazing systems, this is to compensate for the variables in grazing systems that could dilute the effect detected in respiration-chambers. Biochar might still be a valid supplement for cattle for other purposes, for example building soil carbon, but the biochars and doses tested in the current project did not offer methane abatement levels that would allow it to be classified as anti-methanogenic for commercial purposes.

Table of contents

1. Background

Ruminant production systems need new strategies that will improve efficiency and overall profitability. Methane (CH_4) is the main enteric greenhouse gas emitted from livestock and represents between 7 and 18 % of total anthropogenic emissions (Hristov *et al.*, 2013). Methane is a greenhouse gas with a warming potential 28 times greater than CO_2. As such, particularly over the last decade, a deeper understanding and investigations into possible reduction in livestock CH_4 emissions has been targeted by animal nutritionists (Gerber *et al.*, 2013). Enteric methane as an end product of rumen fermentation also represents an energy loss from digested feed for the animal (between 2-12 % of gross energy intake) (Johnson and Johnson, 1995). Microbes known as archaea produce CH_4 in the rumen mainly by reducing C1 compounds with hydrogen (H_2) (among other substrates), thus maintaining a low hydrogen partial pressure within the rumen (Janssen, 2010).

Biochar (i.e. charcoal used in agriculture) has been used as a feed additive in livestock since the 19th century (Totusek and Beeson, 1953) and some producers have been interested in feeding biochar to cattle and sheep because there is evidence that soil health is improved through distribution of biochar in the soil, with help from dung beetles. Recently, it has also gained attention as a possible rumen modifier, particularly in reducing enteric CH_4 emissions in ruminants (Schmidt *et al.*, 2019). However, the effects of biochars on rumen methanogenesis are variable and often contradictory, with some studies showing no effect and other studies reporting a decrease in methane production in ruminants. These effects are likely to depend on the types of biochar and administration regimes and most notably associated dosages. In this project the biochar process was manipulated to produce fit-for-purpose biochar for ruminants and then quantify and validate the effects on animal productivity and methane reduction.

2. Objectives

1. Manipulated the process of biochar production to produce fit-for-purpose biochar for ruminant production.

The project has manipulated biochar production to generate fit-for-purpose biochars to reduce methane emissions in ruminants under controlled feeding conditions. When tested *in vitro*, we demonstrated great variability in the effect on methane production. This was mainly driven by the biomass type used, pre- and post- pyrolysis manipulation and dose rate. There was some evidence that there may have been some influence of or interaction between these characteristics and pyrolysis temperature. The extent of the methane reduction *in vivo* (under controlled feeding conditions) was lower than observed *in vitro*. While a small reduction in enteric methane was detected when selected biochars were fed to cattle under controlled conditions, those same biochar supplements did not decrease methane emissions or improve productivity under grazing conditions.

2. Used a range of different substrates to help generate fit-for-purpose biochar.

Different biomass substrates were used in the project to generate a fit-for-purpose biochar. Fourteen different biochars were generated from seven different biomass substrates and screened using an *in vitro* fermentation batch culture system: *Eucalyptus globulus, E. marginata*; *E. pilularis*; *Eucalyptus spp*; *Acacia cambagei*; *Melaleuca alternifolia;* and straw. Based on preliminary results of the *in vitro* screening, 4 biochars showing greater anti-methanogenic potential were selected for further *in vitro* and *in vivo* testing.

3. Identified a dose that maximises productivity and minimises emissions from livestock systems and that can be incorporated into a palatable livestock diet.

Of the biochar types selected for *in vivo* testing, a dose that decreased methane emissions (8.8-12.9% reduction) in cattle under controlled feeding conditions was identified. However, the same biochars and doses did not decrease methane emissions or increase productivity in subsequent experiments when tested under grazing conditions. Ongoing research is required to identify fit-for-purpose biochars suitable for grazing systems. Successful candidates would require a much greater methane reduction effect under controlled feeding conditions to be suitable as an anti-methanogenic supplement.

3. Methodology

Biochars were selected based on published literature and expertise of the project team. They were sourced either commercially or custom designed within the brief of the current project. The chemical and physical properties of these biochars were examined, and later correlated to the effect on methane. A total of 14 biochars (Appendix 8.1) were first screened *in vitro* in batch cultures as per protocol described previously (Durmic *et al.*, 2014) at a single dose (Experiment 1). In this experiment the highest effective anti-methanogenic dose reported in literature was used to improve the chance of achieving a measurable difference between tested biochars. Five biochars were selected based on this screening that showed either reduced methane production or a related promotion of other fermentative microbial gases. These were tested further in a range of concentrations using the *in vitro* batch culture system to identify the optimal doses; that is the dose that resulted in the highest reduction in methane without affecting other microbial gases, as an indicator of an otherwise unperturbed microbial fermentation (Experiment 2). Based on the results from Experiment 2, four biochar x dose combinations were consequently selected and tested in a longer term, open fermentation system (Rusitec) to confirm that results that were obtained in the 24 hour batch culture, persisted over a longer period of time in an advanced microbial *in vitro* system (Experiment 3).

Based on the results of the Experiment 3 (Rusitec), biochars with 3 different levels of nitrate (NextGenBC) were designed to test the hypothesis that increasing the amount of nitrate associated with the biochar enhances the reduction in methane (Experiment 4). Finally, two biochars (1 x commercial and 1 x custom-made), which showed the most anti-methanogenic potential from Experiments 3 and 4 were tested in the Rusitec (Experiment 5) at 2 levels closer to published and more practical dosages for the subsequent *in vivo* studies (Schmidt *et al.*, 2019).

Based on the results obtained from the sequence of Experiments 1-5, the work progressed to *in vivo* experiments where the two biochar types showing greatest anti-methanogenic potential were tested at three doses in animals. These were fed to cattle in a controlled feeding study in Northern Queensland (*in vivo* Experiment 1), and in grazing studies in both Western Australia (*in vivo* Experiment 2) and in Northern Queensland (*in vivo* Experiment 3). Methane emissions were measured in *in vivo* Experiments 1 and 3, and animal production parameters in *in vivo* Experiments 2 and 3.

3.1 *In vitro* experiments

3.1.1 *In vitro* experiment 1: Screening of biochars in batch culture

A selection of 14 biochars were sourced either commercially or custom-designed as presented in Appendix 8.1. These were initially tested using *in vitro* batch culture to examine their effect on rumen microbial fermentation. In this screening, biochars were added at 20% of substrate (DW), which was comparable to the highest level reported in the literature (Pereira *et al.*, 2014). The testing was conducted as per protocols described earlier (Durmic *et al.*, 2014, Durmic *et al.*, 2010) and the dose and conditions as per (Pereira *et al.*, 2014). Briefly, 0.12 g of biochar was mixed with 0.48 g of fibrous substrate (oaten chaff) in sealed anaerobic serum bottles and microbial methane production was tested. Control cultures consisted of oaten chaff fermentation substrate alone, and each treatment was run in triplicate. Oaten chaff was selected as the fermentation substrate in this study as it is commonly used as major dietary supplement/component of ruminant diets in WA. In addition, it is high in fibre, highly methanogenic and it does not contain plant secondary compounds that may otherwise interfere with rumen methanogenesis or microbial populations. On the day of the experiment, fresh rumen fluid was collected from three rumen-fistulated Merino wethers and strained and buffered to pH 7.0. Serum bottles were then filled with 60 ml buffered rumen fluid inside an anaerobic chamber, sealed and crimped, and incubated with shaking for 24h/39°C. At the end of incubation, total gas produced was measured in the headspace with a pressure transducer and used as an indicator of overall rumen microbial fermentation and activity. A sub-sample of this headspace gas was then run through a gas chromatograph (GC) to measure the relative concentration of methane in the headspace gas mixture.

Statistical analyses in Experiments 1, 2 and 4: All data were analysed using SAS JMP® software and the treatment responses in gas production and methane were examined in separate models with treatment as a factor. Each factor had three experimental units and data were analysed performing one-way analysis of variance and treatment as a fixed effect: $Yij = \mu + Ti + Eij$, where Yij was the observation, μ was the overall mean for each parameter, Ti was the effect of treatment and Eij was residual error. Least significant difference (LSD) was used to compare the treatments to respective controls and significant differences were declared at $P<0.05$.

3.1.2 *In vitro* experiment 2: Dose-response of selected biochars in batch culture

In Experiment 2, four selected biochars; Biochar 3, Biochar 4, Biochar 5, and Biochar 6 were tested at three doses that were lower than the dose applied in Experiment 1: 1 g, 5 g and 10 g per 100 g substrate. Treatments were tested in the same manner as described for Experiment 1.

Statistical analyses in Experiment 1, 2 and 5: All data were analysed using SAS JMP® software and the treatment responses in gas production and methane were examined in separate models with treatment as a factor. Each factor had three experimental units and data were analysed performing one-way analysis of variance and treatment as a fixed effect: $Yij = \mu + Ti + Eij$, where Yij was the observation, μ was the overall mean for each parameter, Ti was the effect of treatment and Eij was residual error. Least significant difference (LSD) was used to compare the treatments to respective controls and significant differences were declared at $P<0.05$.

3.1.3 *In vitro* experiment 3: first Rusitec continuous culture experiment

The Rusitec experiment was conducted to study the selected biochars at different doses that were found to be the most active in reducing methane when tested in the short *in vitro* incubations. Further, these were selected to contrast in their parent material, as well as some inorganic additives, for example nitrates, and alterations like acidification. The biochars and doses selected were Biochar

3 at 5 g/100 g substrate, Biochar 4 at 5 g/100 g substrate, Biochar 5 at 5 g/100 g substrate and Biochar 6 at 1 g/100 g substrate. Two of the selected biochars were obtained from commercial companies (i.e. Biochar 5 and Biochar 6), while two other biochars were custom-designed and manufactured by the project team (Table 1). Biochar composition, manufacture and post-pyrolysis additions are summarized in Table 1 below. Briefly, the two commercial biochars were mainly based on wood waste from *Eucalyptus marginata*, *Melaleuca alternifolia* or *Acacia cambagei*, while custom-made ones from an undefined hardwood, plus wheat straw in Biochar 4. They also contained significant portion of silica minerals such as zeolite and or bentonite, while Biochar 3 also had $FeSO_4$. Pyrolysis temperature also varied, 500°C or below for commercial ones, and 600°C for custom-made ones. One biochar was acidified with HCl, two had glycerol or molasses added, and one had mineral salts and one nitrates in the form of KNO_3.

Table 1. The composition, processes and post-pyrolysis additions of four biochars used in the Rusitec

Detail	Biochar 3	Biochar 4	Biochar 5	Biochar 6
Parent material (g)				
Wood/bark	300	-	-	-
Hardwood	-	200	-	-
Mixed eucalyptus (Eucalyptus marginata) hardwood	-	-	500	-
Tea tree (Melaleuca alternifolia) waste	-	-	250	-
Gidgee wood (Acacia cambagei)		-	-	300
Wheat straw	-	200	-	-
Wheat straw ash	-	100	-	-
Soya bean	-	-	250	-
Zeolite	300	100	-	-
Bentonite	-	100	-	-
$FeSO_4$	90	-	-	-
Pyrolysis temperature °C	600	600	350-500	450
Post-pyrolysis additives (g/100g biochar)				
Acidified	6 (11 M HCl)	-	-	-
Glycerol	20	-	-	-
Molasses	-	-	-	8.5
NaCl	-	-	-	31.5
$CaCO_3$	-	-	-	30
KNO_3	-	3.3	-	-

The *in vitro* continuous fermentation system (Rusitec) was conducted as described by Czerkawski and Breckenridge (1977) and optimized in the UWA laboratory by Ghaffari *et al.* (2014) and Garcia *et al.* (2019). The experiment lasted 14 days, consisting of seven days of an introductory (stabilization) period, followed by 7 days of measurements (Days 8-14). Gas samples were collected daily to measure total gas production and methane, while the fermentation liquid was sampled for analysis of pH, Eh, VFA and NH_3 concentrations, as well as for EMS analysis before introduction of treatments (D7), and at the end of the experiment (D14). On days subsequent to these (D8 and D15), residual digesta left in the feed bags were collected to examine the disappearance of dry matter (DDM) and other chemical analyses, as well as for electron microscopy analysis.

Two separate samples (1 ml) of fermentation liquid were collected directly from the fermentation vessels 3 h after substrate was added, mixed with 200 µL of 25% orthophosphate acid respectively, and stored at −20 °C for analysis of VFA and NH_3 concentration. The determination of VFA concentrations was conducted using an Agilent 7890A GC (Agilent Technologies, Santa Clara, Canada) fitted with a flame ionisation detector (capillary column HP-FFAP (30 m x 0.53 mm x 1.0 micron) with hydrogen as the carrier gas), while NH_3 concentrations were determined by a direct enzymatic method using an Olympus AU400 Auto analyser (Olympus Corporation, Tokyo, Japan).

The residue remaining in the feed bags after 48 h of fermentation was collected, washed under running distilled water and dried at 65 °C for 48 h. The oven-dried residue and the substrate for each treatment were grounded through a 1-mm sieve using a grinder and analysed as per AFIA standard1 for dry matter (DM) and ash, neutral detergent fibre (NDF) and acid detergent fibre (ADF) using Ankom 200/220 fibre analyser (Ankom Technology Co., Macedon, NY, USA). The DM and ash weight was used for calculation of *in vitro* dry matter digestibility (IVDMD) and *in vitro* organic matter digestibility (IVOMD) over a period of 48 h using the formula:

IVDMD (g/kg) = (1 − residue DM/feed DM) × 100
IVOMD (g/kg) = [1 – (residue DM- residue ash) /(feed DM-ash)] × 100
In vitro neutral detergent fibre disappearance (NDFD) was calculated by the following:
NDFD = [1- [(100-IVTDMD) : NDF}] x 100

Where:
NDF = neutral detergent fibre (g per kg of DM)
IVTDMD = *in vitro* true dry matter disappearance (g per kg of DM)

Statistical analyses
The data were analysed using one-way ANOVA with treatments as factors. The interaction between treatments and day was included. The data obtained from D8 and D14 were analysed using repeated measurement using a separate REML procedure, with treatments and days as fixed factors and fermenters as random effect. The interaction between treatments and day was included. When the interaction between treatment and day was significant, simple linear regression with groups (i.e. treatments) was used to estimate the differences between treatments within each period. All statistical analyses were performed using R or JMP statistical software and any P values less than 0.05 were considered as statistically significant.

3.1.4 *In vitro* experiment 4: Screening of NextGen biochars in batch cultures

We tested the hypothesis that increasing levels of nitrates in biochar may augment the CH_4 reduction observed with the custom-made biochars screened in Experiment 2.
A total of 3 NextGenBC were prepared, as described (Table 2). The parent material was mixed and pyrolysed, and where applicable, biochars were acidified. They were then enriched with KNO_3, at four levels – 0 g (no KNO3, NT0), 3.33 g (NT3), 6.67 g (NT6) and 9.99 g (NT9) of KNO_3 per 100 g of biochar. These levels were chosen as increments from the original KNO_3 level in Biochar 4, i.e. 3.3 g per 100 g biochar, as well as having a biochar with no nitrate. We took some caution in choosing the levels, based on reports of nitrate toxicity (Lewis, 1951). Briefly, appropriate amounts of KNO_3 were first dissolved in water and then added to the appropriate amount of biochar to allow saturation of the matrix. Experiment 4a was designed to test biochars where these were dried for seven days at 50 °C (this denoted with letter 'd' at the end), while Experiment 4b was focused on two acidified biochars that were tested after drying for one day at 50°C (denoted with letter 'w' at the end).

The biochars were tested in an *in vitro* batch culture and included at a dose that was found to be most effective for the two custome-made biochars when tested in the first Rusitec experiment.

Table 2. Description of NextGenBC – parent material, temperature and post-pyrolysis manipulations

Detail	Biochar 3.1	Biochar 4.2	Biochar 4.1
Parent material (g)			
Wood/bark	300	-	-
Hardwood	-	200	200
Wheat straw	-	200	200
Wheat straw ash	-	100	100
Zeolite	300	100	100
Bentonite	-	100	100
$FeSO_4$	90	-	-
Pyrolysis temperature (°C)	600	600	600
Post-pyrolysis manipulation (g/100 g biochar)			
Acidified	6 (11 M HCl)	6 (11 M HCl)	-
Glycerol	20	-	-
KNO_3	0 ('NT0), 3.3 ('NT3), 6.6 ('NT6), 9.9 ('NT9)	0 ('NT0), 3.3 ('NT3), 6.6 ('NT6), 9.9 ('NT9)	0 ('NT0), 3.3 ('NT3), 6.6 ('NT6), 9.9 ('NT9)

In experiment 4b, we tested the effect of the drying process of biochars on their effectiveness. This may be of a practical value when producing biochars, as well as storing them longer-term on farm. The hypothesis was that the effect of drying will not diminish even if biochars are dried for a shorter period of time. For this, we focused on two biochars that showed correlations between nitrate level and CH_4 reduction, i.e. acidified versions - Biochar 4.2 and Biochar 3.1 (Table 3).

Table 3. List of treatments, water content and amounts used in experiment 4.

Biochar	Water content (ml/100 g)	Amount of wet biochar (g) added per 0.5 g substrate	KNO_3 concentration (g/100 g substrate)
Biochar 4.2 NT0w	20	0.038	0.00
Biochar 4.2 NT3w	54	0.065	3.33
Biochar 4.2 NT6w	66	0.088	6.66
Biochar 4.2 NT9w	65	0.086	9.99
Biochar 3.1 NT0w	23	0.039	0.00
Biochar 3.1 NT3w	52	0.063	3.33
Biochar 3.1 NT6w	48	0.058	6.66
Biochar 3.1 NT9w	56	0.068	9.99

3.1.5 *In vitro* experiment 5: Second Rusitec experiment assessing dose response

This experiment was conducted to test the selected biochars in the Rusitec system at two doses which were closer to industry practice and included in animal trials (Schmidt *et al.*, 2019). The biochars were tested at two doses – 1 g and 2 g of biochar per 100 g of substrate (oaten chaff). Two biochar types – a custom-made NextGen biochar Biochar 4.2 NT6 (based on original Biochar 4 properties + acidification + KNO_3 increased) and a commercial biochar Biochar 6 were selected for

testing in the second Rusitec experiment. Their selection was based on their strong potential to inhibit methane (*in vitro* experiments 1-4), ability to deliver nitrates (Biochar 4.2 NT6), or commercial availability as a feed additive (Biochar 6).

The two selected biochars differed in parent substrate - *Acacia cambagei* in Biochar 6 and *Eucalyptus globulus*, straw, bentonite, zeolite in Biochar 4.2 NT6, as well as in pyrolysis temperature and post-pyrolysis amendments, resulting in differences in their pH (alkaline vs acidic) and some other basic properties.

For this experiment, a new batch of Biochar 4.2 NT6 was prepared in May 2020. The Biochar 6 for the second Rusitec was sourced from the same batch that was used in the previous *in vitro* trials and stored in a glass bottle at 4°C. Composition and procedures for each biochar are listed in Table 4.

The in vitro continuous fermentation system (Rusitec) was conducted as described by Czerkawski and Breckenridge (1977) and optimized in the UWA laboratory by Ghaffari *et al.* (2014) and Garcia *et al.* (2019). The experiment lasted 22 days, consisting of seven days of an introductory (stabilization) period, followed by 14 days of measurements in two Experimental periods (Experimental period 1, Days 8-14, and Experimental period 2, Days 15-22). In these measurement periods, gas samples were collected daily to measure total gas production and methane, while the fermentation liquid was sampled for analysis of pH, Eh, VFA and NH_3 concentrations, as well as for EMS analysis before introduction of treatments (D7), at the end of Experimental period 1 (D14) and Experimental period 2 (D21). On days subsequent to these (D8, D15 and D22), residual digesta left in the feed bags were collected to examine the disappearance of dry matter (DDM).

Table 4. Composition of biochars used in experiment 5.

Detail	Biochar 4.2 NT6	Biochar 6
Parent material (g)		
Eucalyptus spp.	200	-
Acacia cambagei	-	300
Wheat straw	200	-
Wheat straw ash	100	-
Zeolite	100	-
Bentonite	100	-
Pyrolysis temperature (°C)	600	450, hold time 12h
Post-pyrolysis manipulation (g/100 g biochar)		
Acidified	6 (11 M HCl)	-
KNO_3	6.6	-
NaCl	-	31.5
$CaCO_3$	-	30.0
Molasses	-	8.5
pH	5.7	8.0

Two separate samples (1 ml) of fermentation liquid were collected directly from the fermentation vessels 3 h after substrate was added, mixed with 200 µL of 25% orthophosphate acid respectively, and stored at −20 °C for analysis of VFA and NH3 concentration. The determination of VFA concentrations was conducted using an Agilent 7890A GC (Agilent Technologies, Santa Clara, Canada) fitted with a flame ionisation detector (capillary column HP-FFAP (30 m x 0.53 mm x 1.0 micron) with hydrogen as the carrier gas), while NH_3 concentrations were determined by a direct enzymatic method using an Olympus AU400 Auto analyser (Olympus Corporation, Tokyo, Japan).

The residue remaining in the feed bags after 48 h of fermentation was collected, washed under running distilled water and dried at 65 °C for 48 h. The oven-dried residue and the substrate for each treatment were grounded through a 1-mm sieve using a grinder and analysed as per AFIA standard for dry matter (DM) and ash, neutral detergent fibre (NDF) and acid detergent fibre (ADF) using Ankom 200/220 fibre analyser (Ankom Technology Co., Macedon, NY, USA). The DM and ash weight was used for calculation of in vitro dry matter digestibility (IVDMD) and in vitro organic matter digestibility (IVOMD) over a period of 48 h using the formula:

IVDMD (g/kg) = (1 − residue DM/feed DM) × 100

IVOMD (g/kg) = [1 – (residue DM- residue ash) /(feed DM-ash)] × 100

In vitro neutral detergent fibre disappearance (NDFD) was calculated by the following:

NDFD = [1- [(100-IVTDMD) : NDF}] x 100

Where:

NDF = neutral detergent fibre (g per kg of DM)

IVTDMD = in vitro true dry matter disappearance (g per kg of DM)

Statistical analyses

The data were analysed using one-way ANOVA with treatments as factors. The interaction between treatments and day was included. The data obtained from the experimental period 1 (P1: D8 and D14) and experimental period 2 (P2: Day 20 and Day 22) were analysed using repeated measurement using a separate REML procedure, with treatments and days as fixed factors and fermenters as random effect. The interaction between treatments and day was included. When the interaction between treatment and day was significant, linear regression with treatments was used to estimate the differences between treatments within each period. All statistical analyses were performed using R or JMP statistical software. P values less than 0.05 were considered statistically significant.

3.2 *In vivo* experiments

The experimental protocol complied with the Australian Code for the Care and Use of Animals for Scientific Purposes (eighth edition, 2013) and was approved by CSIRO Animal Experimentation and Ethics Committee (2020-13, 21-06 & 21-07).

3.2.1 *In vivo* experiment 1: Open circuit respiration chamber trial

Twelve steers (*Bos taurus* x *Bos indicus*, mean LW 422 ± 9.9 kg, 3.5 years old) at Lansdown Research Station (Townsville, QLD, Australia) were used in the current study. Animals were randomly allocated to two groups (six animals per group) and weighed every 14 days prior to feeding during the trial. The experimental diet used was a tropical forage offered *ad libitum* (Rhodes grass hay, *Chloris gayana*), chemical composition: DM 907 g/kg fresh matter; in g/kg of DM: CP, 138; NDF, 688; ADF, 375.

Treatments: a commercial biochar: Biochar 6 and a custom-made biochar: Biochar 4.2 NT6 (Table 5) were selected for testing in the *in vivo* experiment based on *in vitro* methane reduction. Biochars were fed to each group of animals as follow:

- Biochar 6 group: received the Biochar 6 mixed with molasses (200 ml) at four levels:
    - 0.0 g biochar/100 g Dry matter intake (DMI)/animal/day (Control period)
    - 0.5 g biochar/100 g DMI/animal/day (Dose 1)
    - 1.0 g biochar /100 g DMI/animal/day (Dose 2)
    - 2.0 g biochar/100 g DMI/animal/day (Dose 3)
- Biochar 4.2 NT6 group: received the Biochar 4.2 NT6 mixed with molasses (200 ml) at four levels:
    - 0.0 g biochar/100 g DMI/animal/day (Control period)
    - 0.5 g biochar/100 g DMI/animal/day (Dose 1)
    - 1 g / 100 g DMI/animal/day (Dose 2)
    - 2 g / 100 g DMI/animal/day (Dose 3)

Biochars were provided to the animals mixed with the hay at two different times: 0 h and 6 h after the feed was offered in an attempt to extend the exposure of the compound in the rumen.

Table 5. Biochar composition and manipulation for animal trials.

Detail	Biochar 6	Biochar 4.2 NT6
Parent material (g)		
Eucalyptus spp.	-	200
Acacia cambagei	300	-
Wheat straw	-	200
Wheat straw ash	-	100
Zeolite	-	100
Bentonite	-	100
Pyrolysis temperature (°C)	450, hold time 12h	600
Post-pyrolysis manipulation (g/100 g biochar)		
Acidified	-	6 (11 M HCl)
KNO_3	-	6.6
NaCl	31.5	-
$CaCO_3$	30.0	-
Molasses	8.5	-
pH	11.43	4.41

Animals were adapted to the diet over a 50-day period, with the last 21 days of the period placed into individual pens for the measurement of intakes and treated with molasses (200 ml/animal/day). On the last two days of that period animals were placed into open-circuit respiration chambers for 48 h to measure enteric CH_4 and H_2 production. Following the initial Control period one group of animals received Biochar 6 at dose 1 and the second group received Biochar 4.2 NT6 at dose 1 for 14 days. On days 13 and 14 of treatment both groups were placed in open-circuit respiration chambers for direct measurement of CH_4 and H_2 production. Doses of both biochars were then increased to the next level (dose 2) for 14 d with CH_4/H_2 measurements in the last 2 days as described previously. At the end of the dose 2 period, biochar levels were increased to dose 3 for 14 d with the same sampling regime in the final two days of the trial.

Rumen fluid and blood samples were collected from the animals at the end of each respiration chamber event (Control, dose 1, dose 2 and dose 3). Rumen fluid samples were collected 3 h post feeding by oesophageal intubation, samples were immediately frozen using dry ice, and stored at −20 °C for ruminal fermentation metabolites or at −80 °C prior to DNA extractions for rumen microbial community composition. Blood samples from all animals were collected by jugular venipuncture using a 10 ml blood Vacutainer tube (BD, Sydney, Australia) containing sodium heparin for plasma and a 10 ml blood Vacutainer tube coated with silica for serum. Both Blood samples for plasma were immediately placed on ice and blood samples for serum were kept for 1 h at room temperature before placing on ice prior to centrifugation. Both blood samples were centrifuged (2500 rpm for 20 min at 4 °C) to separate the plasma and the serum, which were stored at −80 °C for blood urea nitrogen (BUN) analysis.

An estimation of the nitrate content for each dose of Biochar 4.2 NT6 is shown below:

- Dose 1: ~50 g biochar day, contained 2.02 g nitrates/day/animal
- Dose 2: ~100 g biochar day, contained to 4.04 g nitrates/day/animal

• Dose 3: ~200 g biochar day, contained to 8.08 g nitrates/day/animal

Based on this estimation, the maximum level of nitrates fed to the animals were 6 times below the toxic levels of nitrates reported by Benu *et al*. (2018, 2016).

Regarding the polycyclic aromatic hydrocarbons (PAHs) that biochar might contain, a recent study has found that the average daily PAH intake of ruminants suggests that biochar containing <10 mg/kgdw PAHs will not pose an increased risk when applied as a feed additive (Hilber *et al.*, 2019).

Gas measurements

Four open circuit respiration chambers were used to determine CH_4 and H_2 production from individual steers as described by Martinez-Fernandez *et al*. (2016). Briefly, CH_4 and H_2 emissions were performed using a combination of negative pressure (–5 ± 0.14 Pa) in four clear polycarbonate units (23.04 m^3, 3000 L/min air flow). Air samples passed through a chemical drier and were metered through independent rotameters before compositional analysis for CH_4 (Servomex 4100 Servomex Group Ltd. Crowborough, UK) and H_2 (Dräger X-am 5000, Draeger Safety Pacific Pty. Ltd., Notting Hill, VIC, Australia). CH_4 and H_2 production (g) were calculated by averaging individual animal measurements over each 48 h.

Chemical analysis

Feed samples were dried in a forced-air oven at 105 °C to constant weight prior to grinding. Feed samples were ground through a 1 mm sieve before analysis. DM, ash, NDF, ADF, and total nitrogen contents were analysed at the CSIRO Floreat laboratory (Floreat, WA, Australia).

Concentrations of volatile fatty acids (VFAs) (acetate, propionate, n-butyrate, iso-butyrate, iso-valerate and n-valerate) were measured by gas chromatography (GC) as described by Gagen *et al*. (2014). Iso-valerate (3-methyl butyrate) includes 2-methylbutyrate, which co-elutes.

The NH_3-N concentration and BUN were determined by Chaney & Marbach (1962).

DNA Extractions and Illumina MiSeq Sequencing analyses

DNA extractions from rumen samples were performed as described by Martinez-Fernandez et al. (2016). The 16S rRNA gene was used to characterize the microbial populations in the rumen for bacteria (v4 region) (Kozich *et al.*, 2013). Each DNA sample was amplified using the specific primers and a unique barcode combination as described by de Carcer et al. (2011). Amplification products were visualized by performing gel electrophoresis. Product quantities were calculated, and an equal molar amount of each target product was pooled. The pooled target products were run in a 1.5% agarose gel and bands were visualized and excised under blue light trans-illumination. The amplicons were gel purified with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) prior to submission for 2 x 300 bp Illumina MiSeq sequencing (Australian Centre for Ecogenomics, University of Queensland). Paired-end short-read sequence data generated on the Illumina MiSeq was processed using the USEARCH package (Edgar, 2010). De-multiplexed paired-end sequences were first merged prior to sequence quality filtering, followed by denoising (error correction), chimera checking, and clustering of sequences to Amplicon sequence variants (ASVs) (Callahan *et al.*, 2017). Analysis of microbiota diversity and identification of ASVs significantly altered by supplementation or dam was performed in R following the compositional data analysis (Gloor *et al.*, 2017), using packages mixOmics (Rohart *et al.*, 2017), Phyloseq (McMurdie and Holmes, 2013), propr (Quinn *et al.*, 2017), vegan (Oksanen *et al.*, 2019), ALDEx2 (Gloor *et al.*, 2016), and Metacoder (Foster *et al.*, 2017). Taxonomic classification of bacterial ASVs was done using the IDTAXA algorithm implemented in the DECIPHER R package against the SILVA SSU r132 training set (Murali *et al.*, 2018).

Quantitative PCR Analysis

The DNA samples were used as templates for quantifying the abundance of the mcrA gene for total methanogens, and the 16S rDNA for Methanobrevibacter and Methanomassiliicoccaceae family specific. The primers and assay conditions used were previously published by Denman *et al*. (2007), and Huang *et al*. (2016). Real-time PCR (qPCR) analyses were run in quadruplicate from one DNA extraction on an Applied Biosystems™ ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific Inc.). Assays were set up using the SensiFAST SYBR® Lo-ROX reagents (Bioline). Optimisation of assay conditions was performed for primer, template DNA and $MgCl_2$ concentrations. An optimal primer concentration of 400 nM, with a final $MgCl_2$ concentration of 3 mM were used for each assay under the following cycle conditions: one cycle of 50°C for 10 s and 95°C for 2 min 30 s for initial denaturation, forty cycles at 95°C for 15 s and 60°C for 1 min for primer annealing and product elongation. Fluorescence detection was performed at the end of each annealing and extension step. Amplicon specificity was performed via dissociation curve analysis of PCR end products by raising the temperature at a rate of 0.05°C /s from 60 to 95°C. Changes in targeted populations were calculated using a relative quantification calculation and the 2-ΔΔCt method, with the control period used as the calibrator and total bacterial Ct (cycle threshold) values used as the reference value (Livak and Schmittgen, 2001, Denman and McSweeney, 2006).

Statistical analyses

The effect of treatment was analysed for CH_4 and H_2 production, dry matter intake (DMI), live weight (LW), ruminal fermentation metabolites and methanogen abundances. To study the pre-treatment effect and account for the time effect, data from Biochar 6 group, Biochar 4.2 NT6 group and their respective control periods were analysed separately as a univariate repeated-measures analysis of variance using the GLM procedure of SPSS (IBM, version 21.0). Linear, cubic and quadratic components of the response to incremental dose of each biochar were evaluated using polynomial contrasts. To study the effect of the biochars, (Biochar 6 vs Biochar 4.2 NT6) a univariate model using the GLM procedure of SPSS was used, the treatment was considered the fixed effect with the animal as experimental unit. The effect of the treatment was analysed for CH_4, H_2, DMI, LW, ruminal fermentation metabolites and methanogens abundances. Effects were declared significant at $P \leq 0.05$ and P-values between 0.05 and 0.10 were considered as a trend.

3.2.2 *In vivo* experiment 2: Grazing trial measuring productivity (Manjimup, WA)

The objective of the study was to simulate real grazing conditions; therefore, the supplements were offered to the animals as a group and the intakes per animal would have varied during the trial. The property was divided in 3 sections (15 ha each section) and separate experimental groups were grazed in one section each; the sections were considered replicates with similar grass species (perennial and annual pasture mixture) and biomass. Climate statistics during trial (source BoM): May 2021: Lowest average temperature 10.5 °C, highest average temperature 18.2 °C, rain 132.6 mm; June 2021: Lowest average temperature 7.7 °C, highest average temperature 15.5 °C, rain 100.4 mm.

The two selected biochars were offered to cattle at a producer farm (Manjimup, WA) to study the effect on productivity under grazing conditions in South Western Australia. A washout period of two weeks was applied to all animals prior to a supplementation period of two months. Thirty pregnant cows (*Bos taurus*, BW 550 ± 70 kg) and 30 weaners (*Bos taurus*, BW 295 ± 32 kg) were allocated to 3 groups as described below. Groups were created to have similar average weights (non-significant differences between groups), balanced for sex (weaners) and contained the same number of weaners and cows in each group.

Each group received one of the following treatments:

• Control group: Received glycerol (equivalent to 2 kg glycerol/group/every 2 days). Glycerol was selected because it was the by-product available in the region and was used as the attractant by the producer when feeding biochar to his animals (standard practice).

• Biochar 6 group: Received Biochar 6 + glycerol (4.4 kg biochar mixed with 2 kg glycerol/group/every 2 days)

• Biochar 4.2 NT6 group: Received Biochar 4.2 NT6 + glycerol (4.4 kg biochar mixed with 2 kg glycerol/group/every 2 days)

The amounts offered were equivalent to 0.5 -1 g biochar / 100 g DMI / day, which were based on animal trial 1 (respiration chamber experiment) results, which showed a methane production reduction between 8.8-10% under controlled feeding conditions. The amount offered was in line with industry practice, which recommends between 50-100 g/head/day.

Each group received their respective treatment every two days (equivalent to 2 days of treatment) for 2 months following producer standard practice. All animals were able to access the supplement at the same time and the treatment mix was evenly distributed in the troughs to limit variation in intake between animals.

The animals were weighed prior to and at the end of the supplementation to study the effect of the two biochars on animal body weight and average daily weight gain (ADWG).

Statistical analyses

Data from the trial was analysed as a univariate model using the GLM procedure of SPSS (IBM Corp., version 21.0, Armonk, NY, USA). The treatment was considered the fixed effect with the animal as the experimental unit. The effect of treatment (Biochar 6 and Biochar 4.2 NT6) was analysed for body weight (BW) and average daily weight gain (ADWG). Effects were declared significant at $P \leq 0.05$ and P-values between 0.05 and 0.10 were considered as a trend.

3.2.3 *In vivo* experiment 3: Grazing trial measuring productivity and methane emissions (Lansdown Research Station, QLD)

The objective of the *in vivo* experiment 3 was to study the effect of the selected biochars on methane emissions and productivity in cattle fed biochar under grazing conditions. Forty-five heifers (*Bos taurus* x *Bos indicus*, BW 252 ± 57 kg) were grazed together in the same paddock (~45 ha) at Lansdown Research Station in Northern Australia (Townsville, QLD, Australia) and were allocated to the treatments and water points (daily) using a walk over weigher (WOW) with autodrafter. Paddock grasses and legumes composition: *Urochloa sp*, Rhodes Grass (*Chloris gayana*), Bluegrass (*Dichanthium sericium*), Buffel (*Cenchrus ciliaris*) & Spear grass (*Heteropogon contortus*); legumes: Seca Stylo (*Stylosanthes scabra*), Verano (*Stylosanthes hamata*) & Desmanthus (*Desmanthus sp*). Paddock pasture average nutrient composition (g/kg DM): 82 CP, 688 NDF, 411 ADF, 277 hemicellulose and 66 Ash.

Each animal was allocated to one of the three groups (15 animals per group, statistically non-significantly different average weights between groups) and received one of the following treatments:

• Control group: Received molasses (2.86 kg molasses/group/day)

• Biochar 6 group: Received Biochar 6 + molasses (1 kg biochar mixed with 2.86 kg molasses/group/day; equivalent to 66 g biochar/animal/day)

• Biochar 4.2 NT6 group: Received Biochar 4.2 NT6 + molasses (1 kg biochar mixed with 2.86 kg molasses /group/day; equivalent to 66 g biochar/animal/day)

The doses selected were equivalent to 0.5 -1 g biochar / 100 g DMI/day, which were based on animal trial 1 results (respiration chamber experiment), which showed a methane production reduction between 8.8-10%. The amount offered was in line with industry practice, which recommends between 50-100 g/head/day.

All animals were able to access the supplement at the same time and the treatment mix was evenly distributed in the troughs to limit variation in intake between animals. The objective of the study was to simulate grazing conditions; therefore, the supplements were offered to the animals as a group and the intakes per animal would have varied during the trial. Animal body weights were measured at the beginning and end of the trial in the yards and by WOW over 3 months. Enteric CH_4, H_2 and CO_2 production was measured from each individual animal using the Greenfeed Emission Monitors.

Gas measurements

Heifers grazed together in a 45-ha paddock (Lansdown Research Station) with access to two Greenfeed Emission Monitors (GEM) units (C-Lock Inc., Rapid City, SD, USA) (Zimmerman and Zimmerman, 2012, Hammond *et al.*, 2016). The GEM units were placed adjacent to the entrance of the WOW autodrafter to measure daily enteric methane emissions for 2 months. To control the number and duration of methane measurements, GEM provided pellets to each animal with a maximum of 4 feeding sessions/d and at minimum of 5 h between sessions. In each feeding session the maximum quantity of pellets delivered per animal was 175 g (5 drops of approximately 35 g each with 30 s interval between drops). If cattle did not remain to receive the 5 drops in 1 visit, they could make further visits to the GEM in that session until the maximum pellet drops were dispensed. For emission data to be recorded, animals were required to have their head in the unit for at least 2 minutes as detected by a proximity sensor. Air filters on the GEMs were changed weekly and gas sensors were calibrated automatically weekly at night when no cattle were accessing the units. Daily emission estimates (g CH_4, H_2 and CO_2/d) were all calculated using the data provided by C-Lock to generate emission estimates for individual animals on individual days during the measured period.

Statistical analyses

Data from the trial was analysed as a univariate model using the GLM procedure of SPSS (IBM Corp., version 21.0, Armonk, NY, USA). The treatment was considered the fixed effect with the animal as the experimental unit. The effect of treatment (Biochar 6 and Biochar 4.2 NT6) was analysed for body weight (BW), average daily weight gain (ADWG) and CH_4, CO_2 and H_2 production. Effects were declared significant at $P \leq 0.05$ and P-values between 0.05 and 0.10 were considered as a trend.

3.3 Biochar characterisation

The major effect of biochar relates to changes in properties and the microbial community in the biofilms in the Rumen. Biochars were dissolved and fragmented following ingestion. Additionally, the most reactive form of a biochar will be the sub-micron particles. Thus, physical and chemical properties of fresh biochars were characterised, as well as after dissolution and fragmentation. The fragments have colloidal properties with either a positive or negative potential. It is hypothesised that the higher the charged surface, the greater the effect on formation of biofilms, adsorption of organic molecules from the breakdown of the feed, and colonisation by associated micro-organisms. We also hypothesize interaction with extractible organic molecules (especially the humic-like-substances and low molecular weight acids).

In this regard, basic screening tests were undertaken to determine significant differences between the biochars used in the *in vitro* experiments 1 & 2. More detailed testing was conducted on the samples that were used in the *in vitro* experiment 3 (Rusitec), using the standout biochars with

Sample ID's Biochar 3, Biochar 4, Biochar 5, and Biochar 6. In addition, the 2 biochars produced for the *in vivo* experiments were also characterised.

The main tests carried out were:

1. pH, EC, total Carbon and Nitrogen using multi N/C,
2. Scanning electron microscopy (SEM)
3. Raman spectroscopy to determine the aromaticity of the carbon structure and Fourier transform infrared spectroscopy (FTIR) to determine the relative concentration of functional groups,
4. Dissolution of biochar and then filtration using a 0.45-micron filter,
5. Average size and Zeta potential of the filtrate particles. Zeta potential measures the potential difference existing between the surface of a solid particle immersed in a conducting liquid (e.g. water) and the bulk of the liquid). Colloids with high zeta potential (negative or positive) are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate,
6. Liquid chromatography – organic carbon detection (LC-OCD) of the filtered solution to determine type and concentration of water-soluble organic compounds in the biochars,
7. Soluble elements of the filtrate using Inductively Coupled Plasma Mass Spectrometry (ICP-MS),
8. Gas chromatography–mass spectrometry (GC-MS) of organic compounds on the 4 selected biochars,
9. Elemental analysis of the feed chars was conducted using Laser ablation inductively coupled mass spectrometry (LA-ICP-MS) and X-ray fluorescence (XRF),
10. Electron Paramagnetic Resonance (EPR or ESR) was used to detect species that have unpaired electrons, including free radicals and transition metal ions, in 4 finely ground biochar particles.

The methodology for these tests has been published by van Zwieten *et al*. (2010), Archanjo *et al*. (2017), Hagemann *et al*. (2017) and Taherymoosavi *et al*. (2017).

Gas chromatography–mass spectrometry (GC-MS) of organic compounds on the 4 selected biochars was also carried out. Organics were extracted by automated Soxhlet (VELP) in a dichloromethane: methanol 95:5 v:v mixture, Each sample of biochar (0.5 g) was boiled for 1 hr in 40 mL of solvent and rinsed for 1 h in the condensed solvent vapors. The solvent extract was then evaporated to dryness and derivatized using 0.5 mL of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 1 mL acetonitrile for 15 min at 60°C. Analysis was carried out by gas chromatography/mass spectrometry (GC/MS; Agilent, model number 6890N/5973N) in scan mode using temperature programming (oven 170°C, initial hold 5 min, ramp to 300°C at 5°C/min, final hold 10 min) and a 30 m long capillary column with a (5%-phenyl)-methylpolysiloxane phase, 0.25 mm inner diameter, and 0.25 µm film thickness. Compound identification used the NIST98 library.

The unknown mass spectra of the main peaks were matched with the closest known library match. The similarity index (SI) value identified compounds where there was a SI ≥95% to a known compound. Contaminants such as the plasticizers (i.e 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester, siloxanes) were omitted. While this is mainly presence/absence assay and not quantitative the % area from the chromatograms were used as an indication of approximate abundance within the whole sample and compared to other peaks in the same sample.

4. Results

4.1 *In vitro* experiments

4.1.1 *In vitro* Experiment 1: Biochar screening batch culture

At the end of the 24h incubation period, pH in biochar treatments were comparable to Control (Table 6). Nine treatments produced gas that was significantly lower than the Control, however reduction was not greater than 7%, while Biochar 6 demonstrated a small trend to increase gas. Biochar 6 was most effective in reducing methane that was 8.6% lower than Control, but this was accompanied by a significant reduction in fermentation gas (7%). Biochar 5 also produced significantly less methane than Control – 6.0% when expressed as ml/100ml fermentation gas, or 8.0% when expressed as ml/g DMi, but this treatment did not reduce the total gas production.

Table 6. Effect of selected biochars on gas and CH_4 in batch culture systems (24 h incubations).

Treatment	pH	Gas (kPa)		CH_4 (ml/100 mL)		CH_4 (ml/g DMi)	
Control	6.4	138		13.8		31.9	
Biochar 1	6.5	129	*	13.6		29.2	*
Biochar 2	6.4	134		13.7		30.9	
Biochar 3	6.4	136		13.3		30.3	
Biochar 4	6.5	131	*	13.7		29.9	
Biochar 5	6.4	135		13.0	*	29.3	*
Biochar 6	6.4	140		14.0		32.7	
Biochar 7	6.4	135		13.9		31.5	
Biochar 8	6.5	133	*	14.1		31.2	
Biochar 9	6.5	132	*	13.8		30.6	
Biochar 10	6.5	132	*	13.7		30.1	
Biochar 11	6.4	133	*	13.8		30.7	
Biochar 12	6.5	131	*	13.9		30.5	
Biochar 13	6.5	131	*	13.6		29.9	
Biochar 14	6.5	133	*	13.7		30.5	

* Significance: significantly different to Control, $P < 0.05$

There were no effects of 'age' in the biochars that had both fresh and aged version, but some limited effects of alterations and additives were noticed (Table 7). Addition of glycerol or molasses showed some small trends to increase methane, but these were not significant.

Table 7. Effect of selected biochars on gas and CH_4 in batch culture systems (24 h incubations).

Treatment	Gas (kPa)		CH_4 (ml/100 ml)	CH_4 (ml/g DMi)
Biochar 1	129	*	13.6	29.2
Biochar 2	134		13.7	30.9
Biochar 6	140	*	14.0	32.7
Biochar 7	135		13.9	31.5
Biochar 8	133		14.1	31.2
Biochar 9	132		13.8	30.6
Biochar 10	132		13.7	30.1
Biochar 11	133		13.8	30.7
Biochar 12	131		13.9	30.5
Biochar 13	131		13.6	29.9
Biochar 14	133		13.7	30.5

*Significance - differ significantly to other treatments in the same biochar type ($P<0.05$)

Overall, in this preliminary screening, we were able to detect significant differences in gas and methane production when different biochars were included with a fibrous (oaten chaff) based substrate. The most promising biochar was Biochar 5 which caused reduction in methane without affecting overall gas, suggesting a possible specific anti-methanogenic effect, while Biochar 6 showed some trends in promoting fermentation with a slight increase in gas, hence some fermentation-promoting effects. Another interesting candidate was Biochar 1 that caused greater reduction in methane, but this was accompanied with some reduction in gas. 'Age' had no effect on the parameters measured, while in terms of additives and alterations, only modest effects were observed, and just with addition of glycerol and molasses.

Based on the results from this screening study, Biochar 5 and Biochar 6 were selected due to their clear effects on fermentation (gas) or methane. In addition to these, we decided to also include Biochar 3, a biochar that was initially manipulated to target 'fit-for-purpose' objectives, and Biochar 4, as a non-manipulated version of this biochar, to assess the effects of the manipulation. These four biochars were tested in a dose-response experiment to assess the effects of biochars at lower doses of inclusion.

4.1.2 *In vitro* experiment 2: Dose-response of selected biochars

Inclusion of biochars at selected levels effect gas and methane production (Table 8). All treatments significantly reduced methane (18- 32%), with the highest reductions observed at level of 5 g/100 g in biochar 3, 4 and 5, and 1 g/100 g with Biochar 6. Total gas production was also significantly reduced with all the treatments, but did not exceed 20%

Table 8. Dose effect of selected biochars on gas and methane production in batch culture systems (24 h incubations).

Treatment	Gas (KPa)		CH_4 (ml/100 ml gas)		CH_4 (ml/g DMi)	
Control	132	a	13.8	a	31.9	a
Biochar 3 -1	115	b	9.7	fg	20.8	fg
Biochar 3 - 5	117	ab	9.4	g	20.3	fg
Biochar 3 - 10	114	b	10.1	defg	21.5	cdefg
Biochar 4 - 1	104	b	11.5	b	23.4	bcd
Biochar 4 - 5	115	b	9.4	g	20.2	g
Biochar 4 - 10	104	b	11.3	bc	22.8	bcde
Biochar 5 - 1	118	ab	11.1	bcd	21.6	cdefg
Biochar 5 - 5	111	b	9.9	efg	20.8	fg
Biochar 5 - 10	110	b	10.6	bcdef	22.2	bcdef
Biochar 6 - 1	107	b	10.4	cdefg	21.4	defg
Biochar 6 - 5	105	b	10.9	bcde	22.2	bcdef
Biochar 6 - 10	111	ab	11.4	bc	20.9	efg

Significance: within same column, values not sharing same superscript differ significantly ($P<0.05$)

4.1.3 *In vitro* experiment 3: first Rusitec continuous culture experiment

The Rusitec experiment was conducted to study the selected biochars that were found to be the most active in reducing methane when tested in short in vitro incubations. We selected biochars to contrast in their parent material, as well as other alterations such as addition of nitrates and acidification. The biochars and doses selected were Biochar 3 at 5 g/100 g substrate, Biochar 4 at 5 g/100 g substrate, Biochar 5 at 5 g/100 g substrate and Biochar 6 at 1 g/100 g substrate.

Whilst the gas production was not different between diverse types of biochars and doses, differences occurred in their effect on CH_4. Overall, when compared to the Control average daily CH_4 concentrations were significantly decreased ($P<0.05$) with all biochar treatments, with the highest level of reduction with Biochar 6 (36.3 %), Biochar 4 (40.1 %), Biochar 5 (23.4 %), Biochar 3 (18 %) (Table 9).

Table 9. Effect of selected biochars on CH_4 concentration and production using the Rusitec system (14 days incubation).

Treatment	CH_4 concentration (ml/100 ml gas)	SEM	Total CH_4 produced (ml/24h)	SEM
Control	7.53 [a]	1.37	41.0 [a]	3.5
Biochar 3	4.51 [b]	0.81	33.6 [ab]	5.4
Biochar 4	3.82 [b]	0.54	24.2 [b]	2.7
Biochar 5	4.41 [b]	0.60	31.4 [ab]	3.6
Biochar 6	3.46 [b]	0.53	26.1 [b]	2.0

Significance: within each column, values not sharing the same superscript differ (P<0.05).

Overall, there were some minor differences observed between different biochars and doses in terms of digestibility (Table 10). When compared to the Control, there was no difference in any of the digestibility parameters except for ADFD, which was reduced significantly with all biochars except for Biochar 4 (Table 10).

Table 10. Effect of selected biochars on digestibility parameters (g/100 g) using the Rusitec system (14 days incubation).

Treatment	IVDMD	IVOMD	NDFD	ADFD
Control	31.3	32.9	30.1	16.2[a]
Biochar 3	31.0	31.8	29.7	15.9[bc]
Biochar 4	29.3	36.7	31.8	16.8[a]
Biochar 5	31.7	33.9	28.7	15.0[c]
Biochar 6	32.0	29.8	30.0	16.0[b]
SEM	0.5	0.2	0.1	0.1

Significance: within each column and each experimental period, values not sharing the same superscript differ (P<0.05). IVDMD - in vitro true dry matter disappearance, IVOMD - in vitro organic matter digestibility, NDFD - neutral detergent fibre digestibility, ADFD - acid detergent fibre digestibility, SEM – standard error of means

The concentration of total VFA, acetate, propionate, butyrate, acetate to propionate ratio (A:P) and NH_3 concentrations were not significantly affected by biochar treatment (Table 11) compared with the Control.

Table 11. Effect of selected biochars on fermentation parameters using the Rusitec system (14 days incubation).

Treatment	Acetate (mmol/L)	Propionate (mmol/L)	Butyrate (mmol/L)	Total VFA (mmol/L)	A:P	NH_3 (mg/L)
Control	40.7	18.0	9.9	79.5	2.41	271
Biochar 3	44.7	21.6	10.7	88.6	2.26	275
Biochar 4	41.8	17.9	11.3	84.1	2.41	280
Biochar 5	46.5	21.6	12.6	96.1	2.22	274
Biochar 6	42.9	21.4	11.3	87.4	2.18	273
SEM	1.04	1.03	0.42	3.00	0.1	2.98

Significance: within each column and each experimental period, values not sharing the same superscript differ (P<0.05). A:P – acetate to propionate ratio, SEM – standard error of means.

The digestibility and fermentation parameters were not affected by different biochar treatments, however the effects varied over time for some parameters ($P < 0.05$, data not shown). The CH_4 concentrations were not correlated to any of the parameters measured, and the contrasting doses of the biochar did not alter the response.

4.1.4 Experiment 4: Screening of NextGen biochars in batch cultures

Experiment 4a

Overall, the effect on gas production or CH_4 concentrations was not significant for any of the treatments, and relatively small effects were observed on CH_4 reduction (Table 12). Some reduction in CH_4 production occurred with all of these biochars, but only at the two higher levels of KNO_3, with the highest reduction detected with Biochar 3.1 NT6d and Biochar 4.2 NT6d.

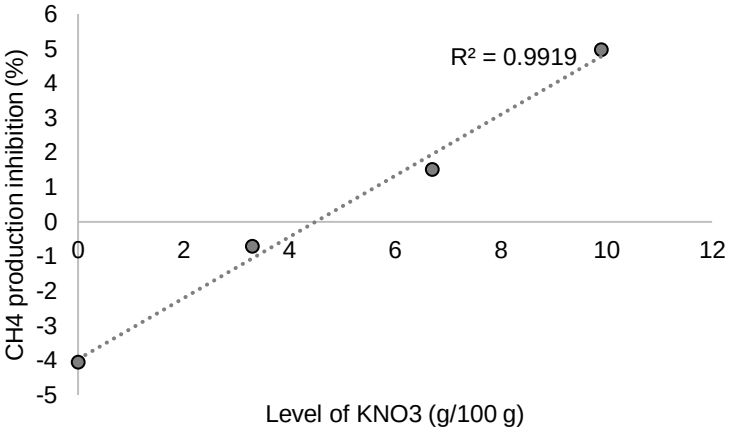
Table 12. NextGenBC effected on gas and CH_4 on gas and CH_4 in batch culture systems (24 h incubations).

Treatment	Gas (ml/g DMi)	CH_4 concentration (ml/100 ml)	CH_4 production (ml/g DMi)
Control	197	10.8	25.2^{ab}
Biochar 4.2 NT0d	216	12.1	26.2^{ab}
Biochar 4.2 NT3d	206	12.1	25.4^{ab}
Biochar 4.2 NT6d	218	11.4	24.8^{ab}
Biochar 4.2 NT9d	193	12.4	24.0^{ab}
SEM	4.9	0.32	0.64
Biochar 3.1 NT0d	208	13.2	27.5^{b}
Biochar 3.1 NT3d	213	12.2	26.0^{ab}
Biochar 3.1 NT6d	208	11.1	23.1^{a}
Biochar 3.1 NT9d	211	11.3	23.7^{ab}
SEM	3.9	0.36	0.58
Biochar 4.1 NT0d	209	12.4	25.8^{ab}
Biochar 4.1 NT3d	176	12.7	25.2 ^{ab}
Biochar 4.1 NT6d	196	12.1	23.5^{ab}
Biochar 4.1 NT9d	208	11.5	24.1^{ab}
SEM	5.8	0.73	0.86

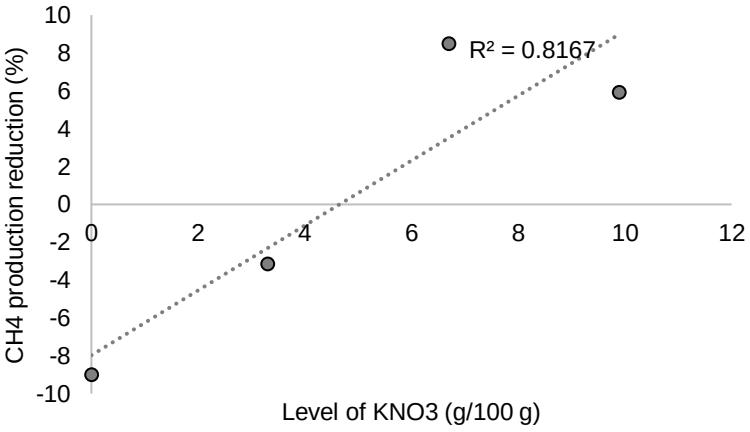
Significance: within each column, values not sharing the same superscript differ ($P<0.05$).

There was a strong dose response between levels of KNO_3 and CH_4 reduction in all three types of biochars, being strongest for Biochar 4.2 NT ($R^2 = 0.99$), followed by Biochar 3.1 NT ($R^2 = 0.82$) and Biochar 4.1 NT ($R^2 = 0.76$, Fig 1). There was a strong linear response to nitrate for Biochar 4.2 (Fig. 1a), but there appeared to be a sigmoidal relationship between level of nitrate and reduction in CH_4 for the other biochars tested (we have only provided the linear trend lines here; Fig. 1b-c). The response (% CH_4 reduction) appears to plateaux and/or peak at NT6 inclusion level.

a) Biochar 4.2 NT



b) Biochar 3.1 NT



c) Biochar 4.1 NT

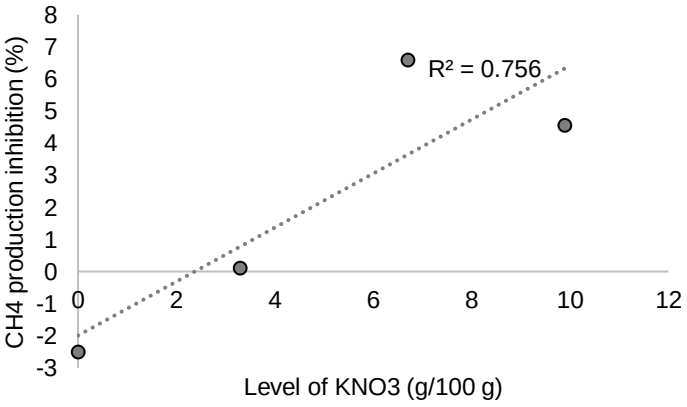


Figure 1. Correlation between KNO_3 level in the biochar and % reduction of CH_4 in treatments compared to Control.

In conclusion, the results suggest a dose response between the level of nitrate in the NextGenBC and CH_4 reduction, despite the effects in batch culture being relatively small. The most promising NextGen biochars were Biochar 3.1 NT6d and Biochar 4.2 6d. It is interesting that the acidified versions had a stronger correlation between level of nitrate and CH_4 reduction, and that the highest level of nitrate was not always the most effective.

Experiment 4b

When compared to the Control, none of the biochar treatments inhibited gas production or caused a significant reduction in CH_4 (Table 13). However, numerically, there was up to 14% (Biochar 4.2 NT9w) or 15% reduction (Biochar 3.1 NT6w) in CH_4 concentrations and production respectively, and there was a very clear relationship between nitrate inclusion and the reduction in CH_4 (Fig. 2 a-b). These reductions in CH_4 are higher than we observed in Experiment 4a, but Experiments 4a and 4b were run two months apart because of the timing of receiving the different biochar sources, and should not be compared directly.

Table 13. NextGenBC effect on gas and CH_4 in batch culture systems (24 h incubations).

Treatment	Gas (ml/g DMi)	CH_4 concentration (ml/100 ml)	CH_4 production (ml/g DMi)
Control	219	13.4	29.4^{ab}
Biochar 4.2 NT0w	218	13.4	29.2^{ab}
Biochar 4.2 NT3w	216	12.7	27.4^{ab}
Biochar 4.2 NT6w	215	11.9	25.7^{ab}
Biochar 4.2 NT9w	218	11.6	25.2^{ab}
Biochar 3.1 NT0w	218	13.9	30.3^{b}
Biochar 3.1 T3w	217	13.4	29.1^{ab}
Biochar 3.1 NT6w	215	11.5	24.8^{a}
Biochar 3.1 NT9w	223	12.4	27.6^{ab}
SEM	1.3	0.3	0.7

Significance: within each column, values not sharing the same superscript differ ($P<0.05$).

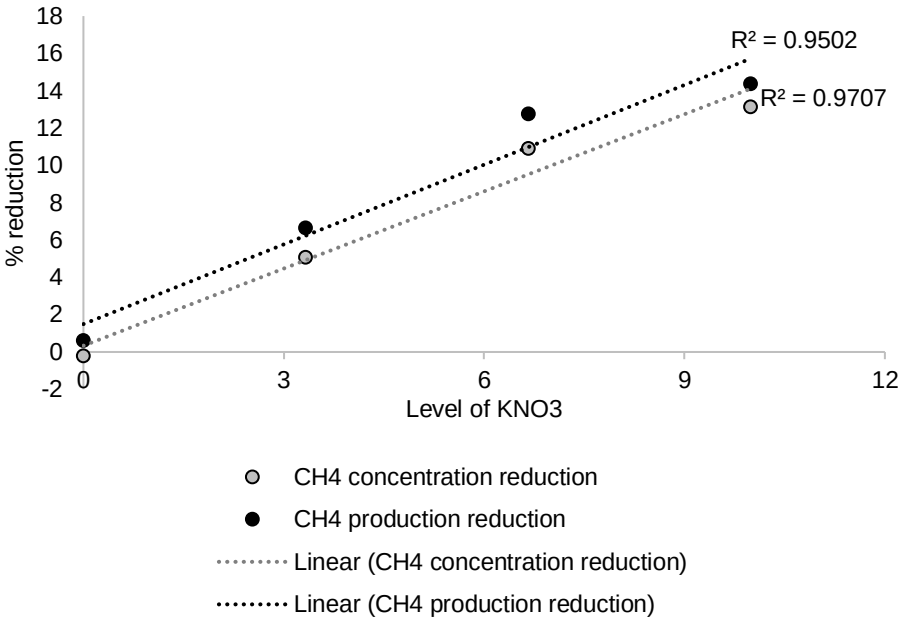
There was a strong positive correlation between the level of KNO_3 and the effect on CH_4 concentration reduction ($R^2 = 0.95$) and production ($R^2 = 0.97$) with Biochar 4.2 NTw biochars (Fig. 2). The correlations were weaker for Biochar 3/NTw biochars - $R^2 = 0.62$ for CH_4 concentration and $R^2 = 0.47$ for CH_4 production. Similar to Experiment 4 a, there was a clear sigmoidal relationship between level of inclusion of nitrate and CH_4 reduction, with plateau or peak at NT6 (we have only included a trend line here). This strengthens the case for there being an optimal inclusion rate of nitrate with biochar, but this needs to be confirmed in the Rusitec so that we can better assess the persistency of the effects on CH_4 production as well as more general long-term effects on other fermentation parameters.

In this experiment, we have shown that the biochars containing nitrates can result in CH_4 reduction, in a dose dependent manner, and without inhibiting microbial activity (gas production). There are some notable differences between the two biochars that may provide further clues and guidance – for example, the one that had stronger correlations contains less zeolite, and has bentonite.

However, given that these mineral materials were not found to influence CH_4 (Varadyova *et al.*, 2003), it seems unlikely that they were independently responsible for the effect.

The reduction of CH_4 in part 'b' of this experiment was higher than that achieved with the biochar that was dried more extensively in Experiment 4a. Whether this effect is significant or not cannot be determined from this dataset because it was not designed to control for batch effects. Regardless of this, the results provided a positive indication that when biochar is applied under field conditions, the moisture content that may have absorbed during storage should not affect, or at least not reduce, its effect on CH_4 production. This means it may not be necessary to dry biochar extensively prior to use.

a) Biochar 4.2 NTw



b) Biochar 3.1 NTw

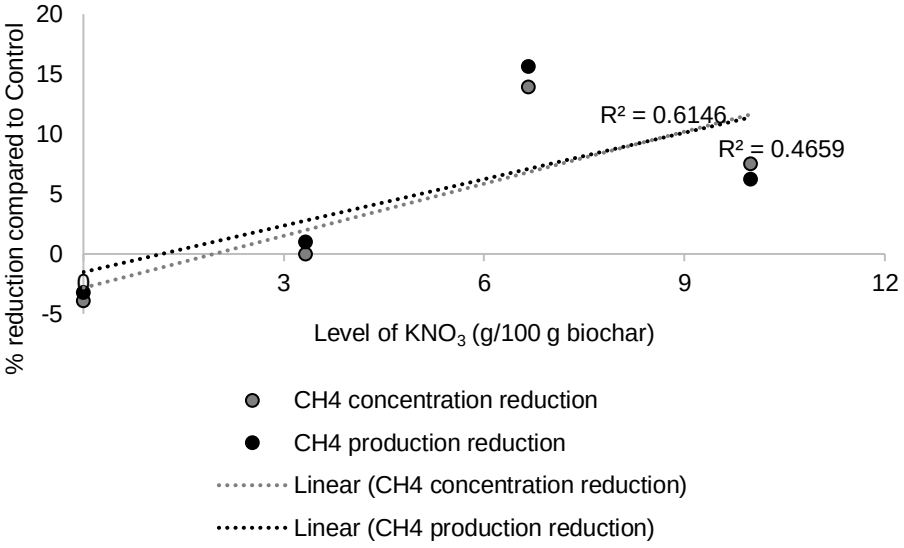


Figure 2. Correlation between KNO_3 level in the biochar and % reduction of CH_4 in treatments compared to Control.

4.1.5 Experiment 5: Second Rusitec experiment assessing dose response

Across both experimental periods, all biochar treatments significantly reduced ($P < 0.05$) methane concentrations and production (Table 14), with Biochar 4.2 NT6 - 1% being the most potent in

reducing methane production (33%) with 1% inclusion. The other treatment (Biochar 6) produced a 23-27% decrease in methane production compared to control with 1% and 2% doses, respectively.

The digestibility parameters were not affected by the addition of biochars when compared to the Control (Table 15), except for ADFD.

Table 14. Effect of selected biochar doses on CH_4 concentration and production using the Rusitec system (22 days incubation).

Treatment	Gas	CH_4 concentration	CH_4 production	
	(ml/24h)	(ml/100 mL gas)	ml/24h	ml/g DMi
Control	1016 [a]	1.46 [a]	14.9 [a]	0.99 [a]
Biochar 4.2 NT6 - 1%	910 [bc]	1.20 [b]	10.0 [b]	0.67 [b]
Biochar 4.2 NT6 - 2%	884 [bc]	1.24 [b]	11.4 [b]	0.76 [b]
Biochar 6- 1%	936 [b]	1.22 [b]	11.5 [b]	0.77 [b]
Biochar 6- 2%	850 [c]	1.24 [b]	10.9 [b]	0.72 [b]
SEM	11.6	0.05	0.67	0.02

Significance: within each column and each experimental period, values not sharing the same superscript differ (P<0.05). DMi – dry matter incubated; SEM – standard error of means

Table 15. Effect of selected biochar doses on digestibility parameters (g/100 g) using the Rusitec system (22 days incubation).

Treatment	DMD	OMD	NDFD	ADFD
Control	38.9	37.6	13.8	12.1 [ab]
Biochar 4.2 NT6 - 1%	39.5	38.3	14.5	12.1 [ab]
Biochar 4.2 NT6 - 2%	39.1	38.1	13.1	10.1 [a]
Biochar 6- 1%	40.0	38.6	15.0	12.8 [b]
Biochar 6- 2%	39.1	38.1	13.4	10.6 [ab]
SEM	0.82	0.80	0.72	0.75

Significance: within each column and each experimental period, values not sharing the same superscript differ (P<0.05). DMD - dry matter disappearance, OMD - organic matter disappearance, NDFD - neutral detergent fibre disappearance, ADFD - acid detergent fibre disappearance, EP – experimental period, SEM – standard error of means

The average daily pH values in the fermentation liquid ranged between 6.64 and 6.77 and were not different between biochar treatments compared to the Control (Table 16). These values were only significantly different between Biochar 4.2 NT6 - 2% and Biochar 6- 1% (P<0.05) .

Across the whole experiment, the addition of biochar did not alter acetate, butyrate, total VFA or A:P, while Biochar 6- 1% significantly (P < 0.05) increased propionate, valerate and A:P.

Table 16. Effect of selected biochar doses on fermentation parameters using the Rusitec system (22 days incubation).

Treatment	pH	Acetate	Propionate	Butyrate (mmol/L)	Valerate	Total VFA	A:P	NH_3 (mg/L)
Control	6.73 [ab]	47.7	22.3 [a]	17.1	7.5 [a]	101	2.2 [a]	232
Biochar 4.2 NT6 - 1%	6.72 [ab]	47.0	23.8 [ab]	15.8	8.4 [ab]	101	2.0 [ab]	241
Biochar 4.2 NT6 - 2%	6.75 [a]	47.7	23.1 [ab]	18.3	7.9 [ab]	103	2.1 [ab]	248
Biochar 6- 1%	6.69 [b]	49.5	26.7 [b]	15.4	8.5 [b]	106	1.9 [b]	239
Biochar 6- 2%	6.73 [ab]	46.7	23.7 [ab]	19.3	8.2 [ab]	104	2.0 [ab]	235
SEM	0.20	1.9	1.2	1.4	0.3	4.3	0.08	7.7

Significance: within each column and each experimental period, values not sharing the same superscript differ ($P<0.05$).
A:P – acetate to propionate ratio, SEM – standard error of means.

4.1.6 Discussion *in vitro*

The results from this *in vitro* study identified engineered fit-for-purpose biochars that consistently reduced methane over 14 and 22 days of incubation using the Rusitec continuous culture fermentation system. We also detected differences in the effects between the types of biochars on pH, gas and methane, which suggests biochar can be modified to achieve 'fit-for-purpose' properties.

We identified four biochar candidates with promising anti-methanogenic effects, which persistently reduced CH_4 over 14 days in a continuous culture Rusitec system (experiment 3). We also detected differences in the effects between the types of biochars on CH_4 and found that none of the biochar types had any detrimental effect on rumen fermentation. Most importantly, we have identified a potential active ingredient, nitrate that can be infused in biochar and result in a dose dependent reduction in CH_4 production. This provides a manipulation based on a mechanistic pathway for reducing methane that is a genuine fit-for-purpose modification.

We demonstrated that the two biochars that showed the most promising anti-methanogenic effect persistently reduced methane over 22 days of incubation in the Rusitec system (experiment 5). The reduction in methane production was in the range of 23-35% compared with the control. This was accompanied with a small reduction in gas production but did not affect digestibility or other fermentation parameters. While the effect on methane did not seem to differ between the different biochar treatments overall, the most potent effect appeared to be with Biochar 4.2 NT6 - 1%, followed by Biochar 6- 1%, Biochar 6- 2% and Biochar 4.2 NT6 - 2%. Increasing the dose of biochar did not seem to improve the effect on methane, but it did affect total gas production, where lower doses of biochar seem to have less of an effect on overall gas production, whilst providing the same level of reduction in methane as the higher doses. There was no-detrimental effect on digestibility, total VFA or NH_3. These findings are in line with the results observed in the first Rusitec experiment (experiment 3) and other published studies, where NH_3 was not affected. In the *in vitro* experiments, the selected biochars did not show any detrimental effect on digestibility, which aligns with published studies using biochars at similar doses, albeit of different parent substrates, that did not have a detrimental effect on digestibility (Teoh *et al.*, 2019, Saleem *et al.*, 2018). However, in these published studies, the effect on methane was not significant, or was rather modest compared to our results.

Our findings (*in vitro* experiments 1-5) support the concept that not all biochars are the same, and that it is possible to manipulate biochar composition and properties to achieve some desired outcomes in ruminant fermentation. In this study, we have confirmed that the two candidates - a commercial and custom designed biochar, had persistent and significant effects on reducing

methane production in the Rusitec (*in vitro*), with no detrimental effect on feed digestibility and microbial fermentation.

An interesting observation was the increase in propionate by Biochar 6. This effect is highly desirable, as propionate is a major gluconeogenic precursor in ruminants (Newbold *et al.*, 2005). This pattern of fermentation along with an increase in branched chain fatty acids, has been reported previously in studies using other anti-methanogenic compounds. The finding aligns with a recent study using pine-based biochars that promoted propionate (Saleem *et al.*, 2018). These findings might indicate that the methane reduction observed was due to a redirection of hydrogen to alternative pathways, but further studies are required to confirm this hypothesis.

The methane inhibition observed in experiment 5 is also in line with published studies. Wood-based biochars at similar doses were found to reduce around 25 % of methane *in vitro* (Saleem *et al.*, 2018), and similar reductions have been reported with straw-based biochars (Cabeza *et al.*, 2018). Small reductions in gas (10-15%) with these types of biochar have also been observed by other authors (Hansen *et al.*, 2012, Cabeza *et al.*, 2018). However, other studies showed that biochar at a dose of 0.5 g per 100 g substrate had a stronger effect on methane (Saleem *et al.*, 2018). For these reasons the dose of 0.5 g of biochar per 100 g of substrate was also included in the *in vivo* trials.

Regarding the custom-made biochar, the original nitrate-enriched Biochar 4 was more potent in the first Rusitec (41% reduction in first Rusitec). The greater effect on methane might be associated to the higher dose used in experiment 3 (5 g biochar/100 g substrate) compared with the doses (1 and 2 g biochar/100 g of substrate) used in experiment 5.

Biochars with acidic pH were associated with greater anti-methanogenic activity as reported by Saleem et al. (2018), it might suggest that the lower pH is linked with some properties that resulted in greater bioactivity. Future experiments should study the effect of pH on bioactivity, as well as the effect of other sources of variability.

In conclusion, we have confirmed that two types of biochars – a commercial biochar (Biochar 6) and an engineered, custom-made, biochar (Biochar 4.2 NT6), included at either 1 g or 2 g /100 g of a substrate relatively high in ADF and NDF and low in DMD can cause a persistent and significant reduction in methane, with only small reductions in total gas, and no detrimental effect on digestibility and VFA concentrations in the Rusitec. There was no significant difference in methane production between the biochar types or their doses, but the reduction in methane was slightly greater in the lower dose of the customed-made biochar: Eucalyptus/wheat/zeolite/bentonite-based, pyrolysed at higher temperature and nitrate-enriched.

4.2 *In vivo* experiments

4.2.1 *In vivo* experiment 1: Open circuit respiration chamber trial

The two biochars tested in the *in vitro* experiment 5 (Rusitec) were selected to progress to the *in vivo* evaluation. Based on the Rusitec results and recent literature (Schmidt et al., 2019), three doses (for each biochar) have been selected for the *in vivo* trial: at 0.5 g (Dose 1), 1 g (Dose 2), and 2 g (Dose 3) per 100 g of DMI/animal/day

No detrimental effects were observed on DMI or rumen fermentation parameters with any of the doses of the biochars.

Methane production (g/day and g/kg DMI Table 17) was decreased significantly ($P < 0.05$) on both biochars compared with the control period. Only Biochar 4.2 NT6 showed a linear and cubic effect, indicating a dose-dependent response. Biochar 6 decreased CH_4 production (g/kg DMI) by approximately 8.8 to 10.0 % and Biochar 4.2 NT6 by approximately 9.5 to 12.9 % compared with

their respective control periods. No significant differences in the amount of expelled H_2 were observed between controls and treatments. In addition, BW, DMI, CH_4 and H_2 production were not significantly different between biochars.

Table 17. Dose effects of Biochar 6 and 2 on DMI, CH_4 and H_2 production in steers feed Rhodes grass hay.

	Biochars	Control	Dose 1	Dose 2	Dose 3	SEM	P-value	Polynomial Contrast
DMI (kg)	Biochar 6	7.91	8.08	8.03	7.90	0.13	n.s.	n.s.
CH_4 (g/day)		189^{a}	176^{bc}	182^{ab}	170^{c}	5.09	0.007	*C*
H_2 (g/day)		0.00	0.00	0.00	0.00	0.00	n.s.	n.s.
CH_4 (g/kg DMI)		23.9^{a}	21.8^{b}	22.8^{ab}	21.5^{b}	0.40	0.029	n.s.
DMI (kg)	Biochar 4.2 NT6	8.54	8.61	8.65	8.47	0.33	n.s.	n.s.
CH_4 (g/day)		197^{a}	180^{bc}	188^{b}	170^{c}	6.89	0.001	*L, C*
H_2 (g/day)		0.00	0.00	0.00	0.00	0.00	n.s.	n.s.
CH_4 (g/kg DMI)		23.2^{a}	21.0^{c}	21.7^{b}	20.2^{c}	0.86	0.001	*L, C*

Polynomal Contrast: Significant (P < 0.05) linear (L) or cubic (C) effects if the response to incremental doses of biochar estimated by polynimal contrast. n.s.: no significant

Regarding the fermentation parameters, no significant effects were observed between control periods and biochar doses for the VFA profile, ammonia concentrations, rumen pH and redox potential. The only significant difference was an increase in blood urea nitrogen for the second dose of both biochars compared with control (Tables 18 and 19).

Table 18. Control and Biochar 6 dose effects (1, 2 and 3) on rumen fermentation parameters and blood urea nitrogen (BUN) in steers fed Rhodes grass hay at 3 h post feeding.

	Control	Dose 1	Dose 2	Dose 3	SEM	P-value	Polynomial Contrast
Rumen pH	7.14	7.12	6.93	7.18	0.07	0.335	n.s.
Redox potential (mV)	-292	-290	-304	-237	10.8	0.086	n.s.
BUN (mg/100 ml)	23.9^{b}	24.0^{b}	27.2^{a}	24.6^{b}	0.92	0.050	*L, C*
Ammonia-N (mg/100 ml)	12.4	12.9	12.0	11.3	0.18	0.727	n.s.
Total VFA (mM)	62.2	50.7	58.7	57.0	3.56	0.081	n.s.
(%)							
Acetate	74.2	74.5	74.5	75.3	0.44	0.737	n.s.
Propionate	13.6	14.2	14.1	14.1	0.11	0.308	n.s.
iso-Butyrate	1.38	1.35	1.36	1.43	0.02	0.226	n.s.
n-Butyrate	6.50	6.61	6.85	6.25	0.18	0.133	n.s.
iso-Valerate	1.45	1.16	1.10	1.16	0.06	0.402	n.s.
n-Valerate	1.37	0.91	1.00	0.86	0.10	0.353	n.s.
n-Caproate	1.46	1.31	1.12	0.95	0.11	0.543	n.s.
ratio A:P	5.46	5.26	5.30	5.37	0.06	0.538	n.s.

Polynomal Contrast: Significant (P < 0.05) linear (L) or cubic (C) effects if the response to incremental doses of biochar estimated by polynimal contrast. n.s. : no significant

Table 19. Control and Biochar 4.2 NT6 dose effects (1, 2 and 3) on rumen fermentation parameters and blood urea nitrogen (BUN) in steers fed Rhodes grass hay at 3 h post feeding.

	Control	Dose 1	Dose 2	Dose 3	SEM	P-value	Polynomial Contrast
Rumen pH	7.04	6.96	7.21	7.06	0.04	0.452	n.s.
Redox potential (mV)	-298	-283	-301	-267	5.76	0.124	n.s.
BUN (mg/100 ml)	24.5^{c}	25.4^{b}	27.9^{a}	24.0^{c}	0.53	0.001	*Q, C*
Ammonia-N (mg/100 ml)	13.8	15.3	14.1	11.4	0.84	0.191	n.s.
Total VFA (mM)	63.1	59.9	48.7	56.2	1.68	0.112	n.s.
(%)							
Acetate	75.8	75.3	75.0	75.5	0.2	0.581	n.s.
Propionate	13.4	13.9	13.5	13.5	0.11	0.718	n.s.
iso-Butyrate	1.33	1.40	1.49	1.46	0.05	0.069	n.s.
n-Butyrate	6.33	6.20	6.64	6.26	0.2	0.341	n.s.
iso-Valerate	1.12	1.18	1.19	1.17	0.05	0.719	n.s.
n-Valerate	0.97	1.07	1.03	0.91	0.03	0.095	n.s.
n-Caproate	1.06	1.03	1.12	1.15	0.05	0.878	n.s.
ratio A:P	5.65	5.45	5.55	5.59	0.05	0.796	n.s.

Polynomal Contrast: Significant ($P < 0.05$) cubic (C) or quadratic (Q) effects if the response to incremental doses of biochar estimated by polynimal contrast. n.s. : no significant

Using sparse PLS Discriminant Analysis (sPLS-DA), ASV's that best characterised the animal treatment groups were determined (Cao *et al.*, 2011), it showed some separation between supplemented and un-supplemented animals for rumen bacteria (Fig. 3 and 4). Clustered image heatmaps (mixOmics analysis) for the selected rumen bacterial ASV's at 0, 0.5, 1 and 2 g of each biochar/kg DMI showed a distinct microbial signature for both biochars compared to control (Fig. 5 and 6). The bacterial ASVs positively associated with Biochar 6 (Fig. 5) classified to the family *Rikenellaceae*, *Christensenellaceae* for the 3 doses and genera *Candidatus Saccharimonas* for the lowest and mid doses used. The bacterial ASVs positively associated with Biochar 4.2 NT6 (Fig. 6) classified to the family *Prevotellaceae* (particularly for the lowest dose), *Christensenellaceae*, *Rikenellaceae* and *Ruminococcaceae*.

Quantitative PCR analysis of the effect of biochars on the abundance of methanogens, *Methanobrevibacter spp*. and *Methanomassiliicoccaceae* family are shown in Fig. 7 and 8. No significant differences were observed when biochars were fed to the animals compared with control periods.

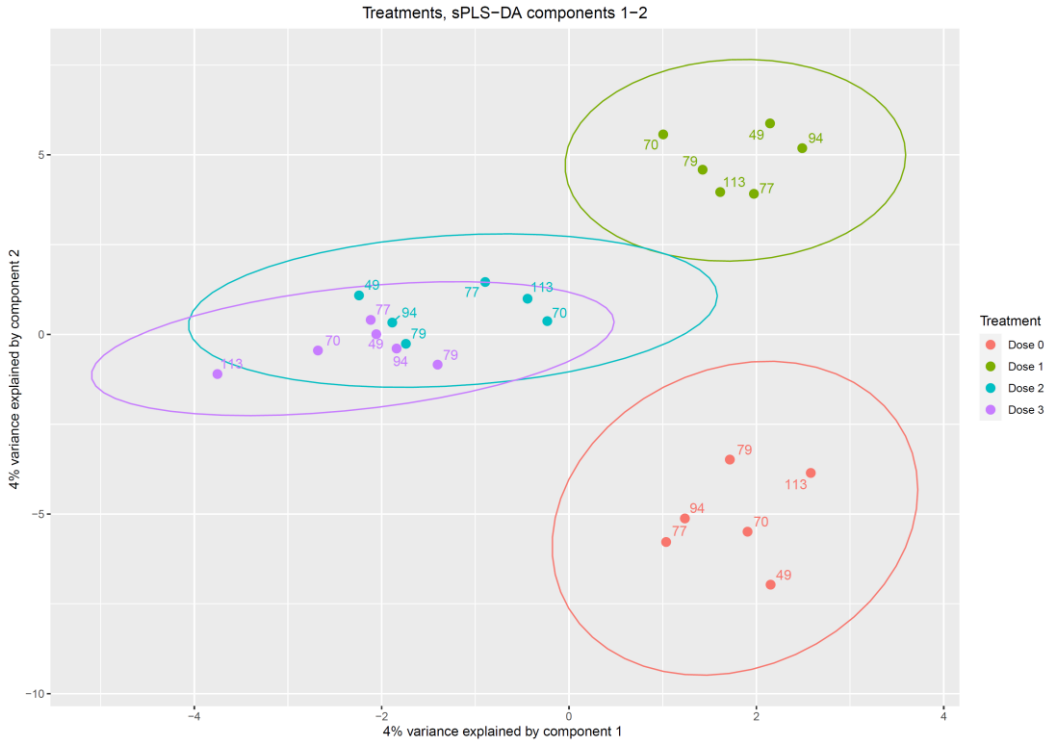


Figure 3. Supervised analysis with sPLS-DA on rumen bacteria community for cattle supplemented with Biochar 6

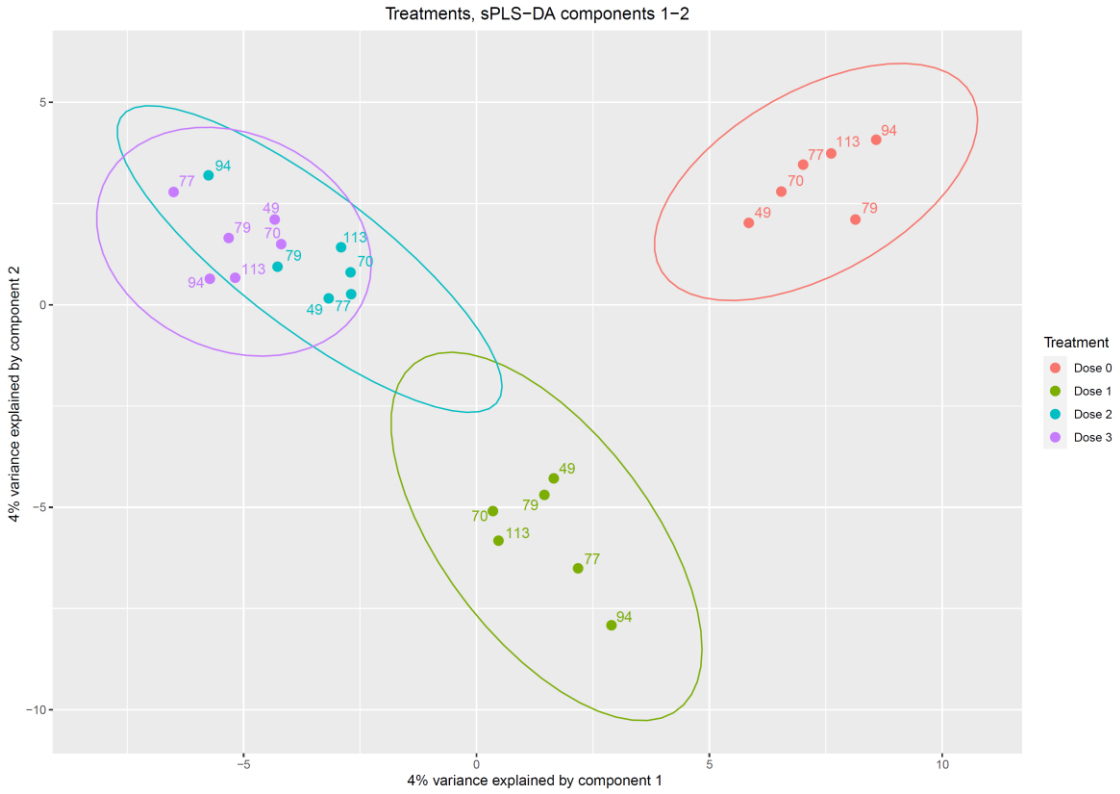


Figure 4. Supervised analysis with sPLS-DA on rumen bacteria community for cattle supplemented with Biochar 4.2 NT6.

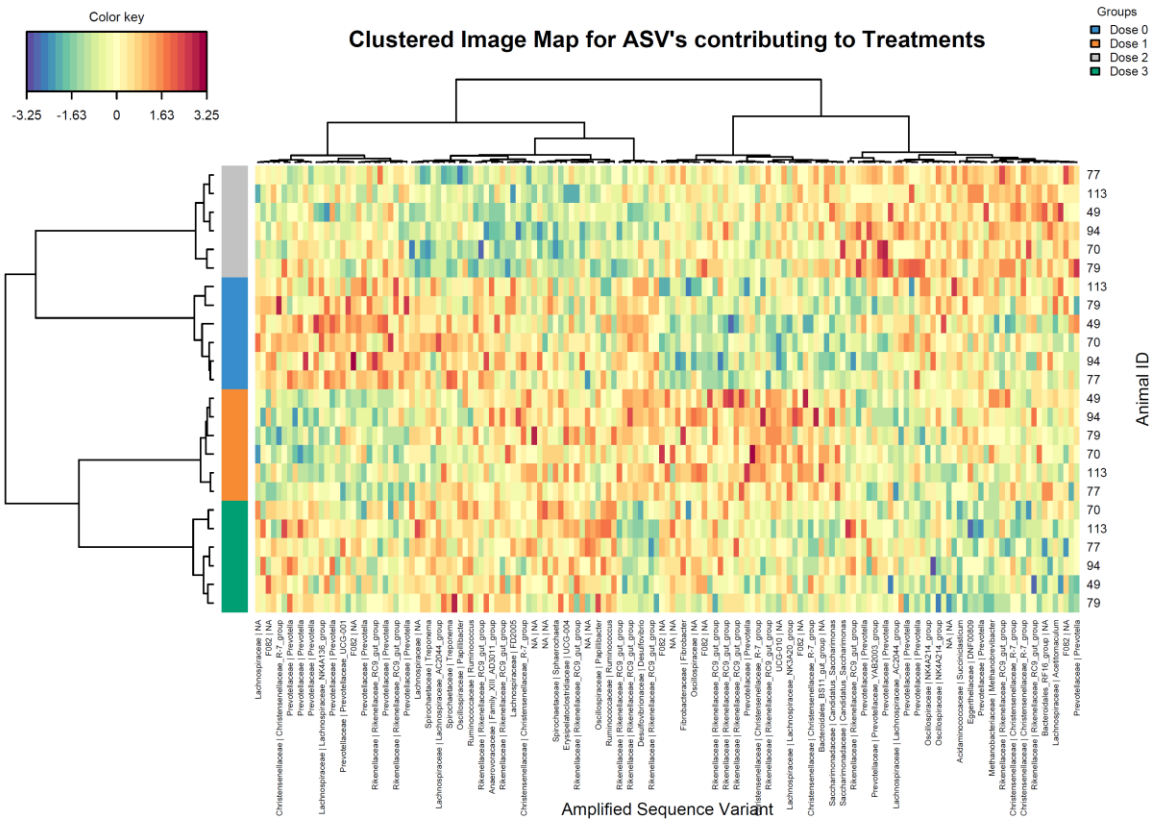


Figure 5. Clustering analysis using a heatmap based on the bacterial ASVs that best characterised the animal supplemented with Biochar 6.

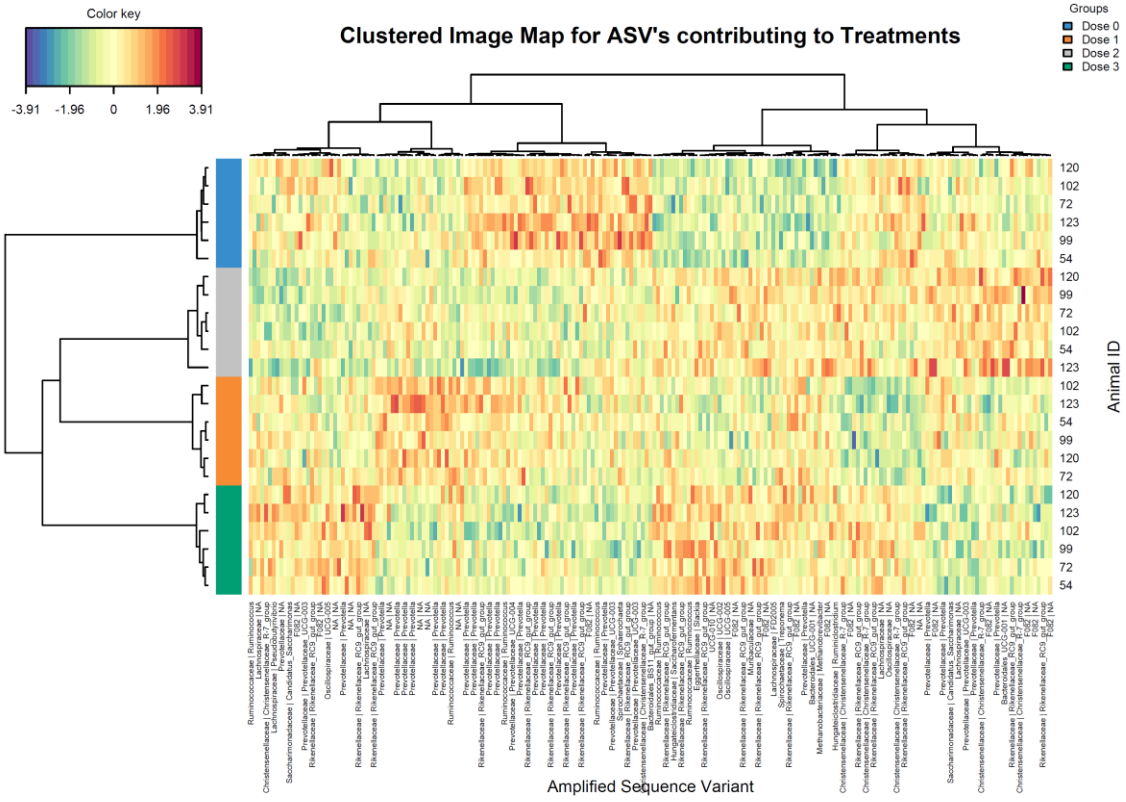


Figure 6. Clustering analysis using a heatmap based on the bacterial ASVs that best characterised the animal supplemented with Biochar 4.2 NT6.

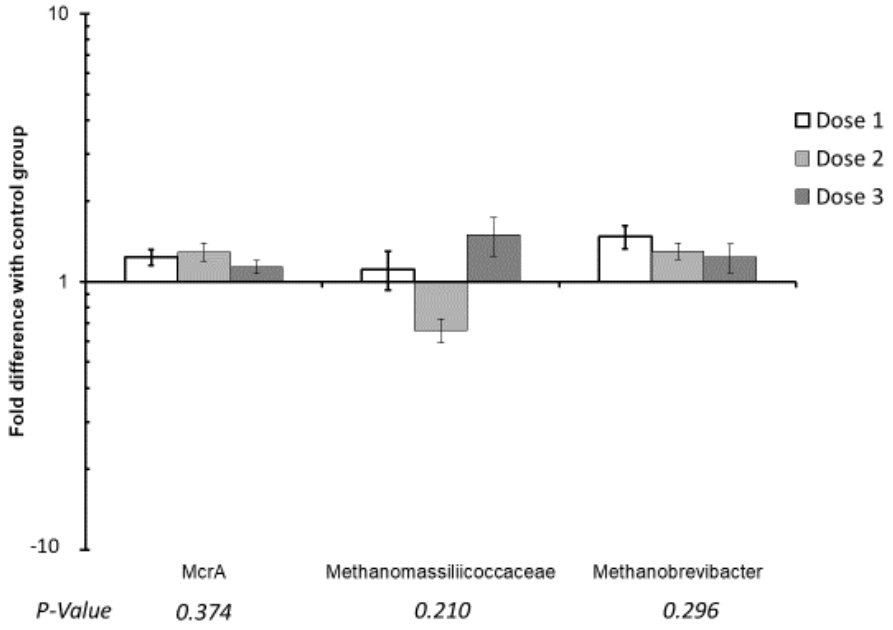


Figure 7. Quantitative PCR (qPCR) analysis of mcrA gene (methanogens), *Methanobrevibacter* spp. and *Methanomassiliicoccaceae* family population changes in response to the three doses of biochar 6. The y-axis denotes fold change from control period.

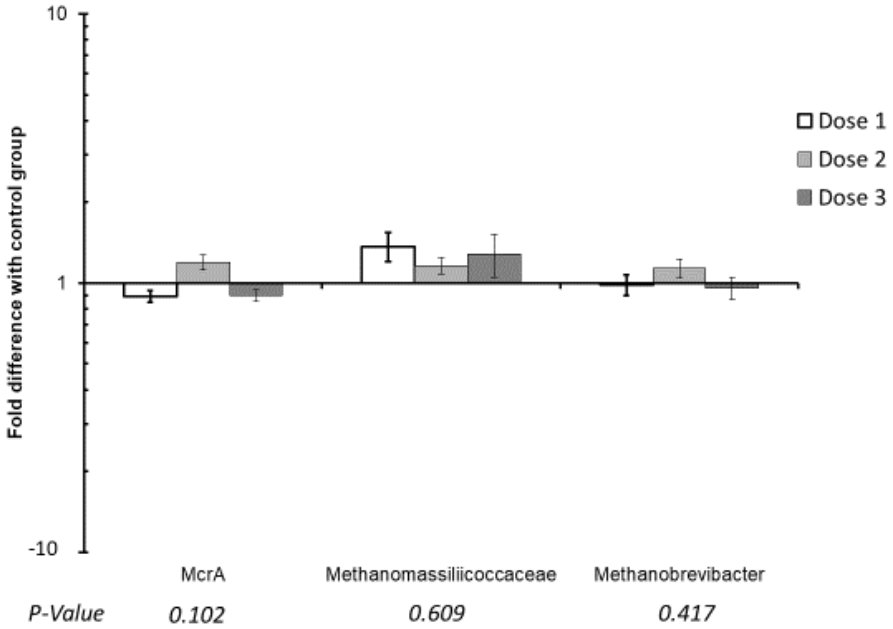


Figure 8. Quantitative PCR (qPCR) analysis of mcrA gene (methanogens), *Methanobrevibacter* spp. and *Methanomassiliicoccaceae* family population changes in response to the three doses of Biochar 4.2 NT6. The y-axis denotes fold change from control period.

4.2.2 In vivo experiment 2: Grazing trial measuring productivity (WA)

The first grazing trial to measure productivity was completed at a commercial farm (Manjimup, WA) and the results are reported in Table 20. No significant differences in body weight were detected between the control and biochars groups in either the cows or weaners before or during the supplementation. The ADWG during the supplementation was significantly higher in control and Biochar 6 compared with Biochar 4.2 NT6. However, ADWG was not significantly different between control and Biochar 6. The lower ADWG observed in Biochar 4.2 NT6 group might be due to the supplement per se, however external factors such as paddock differences or pregnancy stage could have contributed to this result.

Table 20. Body weight and ADWG changes in pregnant cows and weaners supplemented with two biochars during 60 days under grazing conditions in South Western Australia.

	Control	Biochar 6	Biochar 4.2 NT6	SEM	P-value
BW (kg) Cows prior supplementation	554	550	544	14.3	0.964
BW (kg) Cows end of supplementation	575	571	548	14.5	0.740
ADWG (kg) Cows during supplementation	0.340[a]	0.332[a]	0.061[b]	0.04	0.034
BW (kg) Weaners prior supplementation	296	295	295	6.03	0.994
BW (kg) Weaners end of supplementation	307	313	299	5.32	0.546
ADWG (kg) Weaners during supplementation	0.181[ab]	0.297[a]	0.067[b]	0.04	0.036

4.2.3 *In vivo* experiment 3: Grazing trial measuring productivity and methane emissions (Lansdown Research Station, QLD)

The second grazing trial to measure productivity and methane emissions was completed at Lansdown Research Station (QLD) and the preliminary results are reported in Table 21. No significant differences in BW and ADWG were detected between control and biochars groups. In addition, CH_4, CO_2 and H_2 production (g/day) during the supplementation period were not significantly different between the control animals and the animals supplemented with the biochars.

Table 21. Body weight, ADWG, CH_4, H_2 and CO_2 production changes in heifers supplemented with two biochars for 60 days under grazing conditions in Northern Australia (dry season).

	Control	Biochar 6	Biochar 4.2 NT6	SEM	P-value
BW (kg) prior supplementation	251	251	254	8.72	0.983
BW (kg) end supplementation	265	262	265	7.69	0.982
ADWG (kg) during supplementation	0.161	0.134	0.127	0.02	0.711
CH_4 (g/day) during supplementation	181	180	185	6.66	0.866
H_2 (g/day) during supplementation	0.54	0.54	0.51	0.07	0.944
CO_2 (g/day) during supplementation	4181	4007	4190	171	0.711

4.2.4 Discussion *in vivo*

Published studies about biochar effect on enteric CH_4 production in ruminants (*in vivo*) are variable and often contradictory, which might be due to differences in the properties of the biochars used, material source, dose, diet and metabolites that biochars contained. Terry et al., (2019) did not find any significant effect of three doses (0.5 , 1.0, or 2.0 % DMI) of pine enhanced biochar on methane production in cattle fed a barley-silage diet. Similarly, Winders *et al*. (2019) did not find any inhibition in methane production in steers on finishing diets (feedlot) but reported a 10% methane reduction in steers fed growing diets when 0.8 % biochar was supplemented.

In contrast, Leng et al. (2012) found a 20% reduction in methane concentration when 0.6% of biochar was added to the diet of cattle and 40% reduction when biochar was combined with 6% potassium nitrate. However, the methodology used to measure methane emissions in this study did not adhere to the accepted standards and might have generated inaccurate results. For instance, only the concentrations at short periods of time were measured (24 h gas production was not measured in this study), which might have overestimated the methane reduction. Similarly, Al-Azzawi et al. (2021) tested 0.5% DMI of a high-activity microporous of powdered activated biochar in dairy cows and reported a 30-40% reduction in methane concentration. However, as occurred with the study by Leng et al. (2012), the methodology used to measure the methane emissions was not conventional and did not adhere to the accepted standards which might have led to inaccurate results. Furthermore, the enteric methane concentration was measured during short periods of time while animals were in the milking shed, thus the real methane production abatement could not be estimated accurately. Therefore, the methane reduction reported in both studies (Leng *et al.*, 2012, Al-Azzawi *et al.*, 2021) is likely to have been overestimated and further research, using a more accurate and conventional technique for methane measurements, will be required to confirm the real enteric methane reduction in ruminants of these 2 biochars.

The results in this report (*in vitro* and *in vivo*) support the concept that not all biochars are the same, and that might be possible to manipulate biochar composition and properties to achieve some desired outcomes in ruminant fermentation. Based on the rumen fermentation and microbial results, the methane inhibition observed (controlled feeding study and *in vitro* experiments) is likely to be produced by an indirect inhibition of methanogenesis rather than direct action on the methanogens themselves.

The smaller methane reduction observed in the first *in vivo* trial as compared with short-term and long-term *in vitro* experiments have been observed with other anti-methanogenic compounds. The direct extrapolation of the doses from *in vitro* to *in vivo* systems represent a challenge due to several factors, such as the complexity of the rumen microbial community and the rumen fluid passage rate in the animal compared with the *in vitro* systems (Soto *et al.*, 2012). Martinez-Fernandez *et al*. (2013, 2015), reported a 33 % and 48% methane reduction when an organosulfur compound was tested in short and long-term *in vitro* incubations. However, when the same compound and doses were tested in small ruminants (Martinez-Fernandez *et al.*, 2014), did not find a significant reduction in enteric CH_4 production, and only a 10% numerical reduction was reported.

The lack of effect of both biochars in cattle under grazing conditions did not align with the results obtained in the first animal trial under more controlled feeding conditions. This contrast could be explained by the greater impact and variability of feed, herd, animal intake and management in extensive grazing conditions vs intensive livestock systems. Therefore, a successful biochar would require a much greater enteric methane reduction under controlled feeding conditions (than the reduction achieved in the current project) to be suitable as a cost effective anti-methanogenic supplement in a grazing system.

The uptake and effectiveness of anti-methanogenic supplements will be greater in production systems where delivery of feed is more controlled, like in feedlots and dairy farms. However, the

majority of Australian beef industry is pasture and rangeland base (Greenwood *et al.*, 2018), thus the development of new delivery technologies for anti-methanogenic supplements is critical to deliver significant methane abatement in these systems.

4.3 Biochars characterisation

A total of 12 biochar samples were analysed. Based on the results from the batch culture experiments further analyses were performed on the biochars tested in the Rusitec (*in vitro* experiment 3): Biochar 3, Biochar 4, Biochar 5, and Biochar 6. This report has focused on a more detailed characterisation of samples of these 4 biochars in an attempt to in elucidate why they had a greater reduction in methane under *in vitro* conditions.

4.3.1 Basic properties of biochars

The basic properties of 12 biochars tested in experiment 1 are given in Table 22. It was observed that the pH of most of the biochars were classed as base except for the strong acid treated samples and Biochar 4, which had a pH value ranged between 4.2 and 6.5. Biochar 12 was neutral, which probably indicates that the particular sample had a short residence time in the reactor. The electrical conductivity (EC) for the different biochars reveals that Biochar 6 had the added lime and the NaCl and the HCl treated biochar had a high concentration of soluble salts. Similar EC was observed for Biochar 4. Biochar 4, Biochar 1 and Biochar 5 had a relatively high EC, but lower than Biochar 9.

Table 22. Basic properties of the biochars tested in experiment 1.

Biochar	pH	EC (mS/cm)	N (%)	C (%)	H (%)	S (%)
Biochar 1	8.2	0.118	0.10	47.00	5.209	0.250
Biochar 2	2.7	1.4	0.04	46.57	4.248	0.169
Biochar 3	4.2	12.4	0.55	11.62	0.67	2.02
Biochar 4	9.6	1.2	0.19	17.37	1.06	0.07
Biochar 5	7.3	2.7	0.71	61.45	3.484	0.510
Biochar 6	8.7	Higher than detection limit	1.01	24.78	2.894	0.201
Biochar 7	6.3	9.0	0.64	47.01	2.823	0.110
Biochar 8	7.3	0.756	0.70	51.28	2.318	0.246
Biochar 9	6.5	0.896	0.62	67.12	3.035	0.156
Biochar 11	8.7	0.462	0.45	74.18	2.829	0.098
Biochar 12	6.9	0.422	0.56	80.21	1.933	0.095
Biochar 13	8.7	0.136	0.67	78.48	1.862	0.142

The basic properties of the 4 selected biochars are shown in Table 23. The pH of three of the biochars was strongly basic, while acid-treated biochar Biochar 3 had a pH of 4.2. The highest electrical conductivity (EC) was measured for Biochar 6, a formulation that had additional mineral salts. Biochar 3 also had a relatively high EC, indicating that the HCl solubilised some of the minerals in the biochar and the glycerol. A similar EC was observed for the other two biochars.

The nitrogen content varied between samples and was the highest in Biochar 6 and Biochar 5, which was likely to be due to a significant portion of leafy plant material in the parent material. These two also had a carbon content typical of biochars made from agricultural residues, while the other two

had lower N and C, but higher minerals, which is consistent with their parent material. The sulphur content was much higher in Biochar 3 than the other biochars, which was due to the addition of $FeSO_4$.

Table 23. Basic properties of the biochars tested in experiment 2 and 3. EC - electrical conductivity, N – nitrogen, C – carbon, H – hydrogen, S- sulphur.

Biochar	pH	EC (mS/cm)	N	C	H	S	Minerals
			(g/100 g)				
Biochar 3	4.2	12.4	0.55	11.62	0.67	2.02	85.14
Biochar 4	9.6	1.2	0.19	17.37	1.06	0.07	81.31
Biochar 5	7.3	2.7	0.71	61.45	3.48	0.51	33.85
Biochar 6	8.7	nd	1.01	24.78	2.89	0.20	71.12

4.3.2 Surface area, Colloidal particle size and Zeta potential

Table 24 shows that the average particles size of all the biochars was less than 300 nm, with the smallest value observed for Biochar 3. the Biochar 3, on the other hand, had the highest positive zeta potential, followed by Biochar 4. Biochar 11 and Biochar 5 sample showed the highest negative zeta potential.

Table 24. Average particle size and Zeta Potential (mean ± SEM) of the biochars tested in experiment 1.

Biochars	Average particle size (nm)	Zeta potential (mV)
Biochar 1	176±0.65	-31.4±1.36
Biochar 3	132±4.46	-85.6±9.07
Biochar 4	153±8.51	-73.1±1.89
Biochar 5	219±14.45	-50.4±2.65
Biochar 6	281±60.59	-8.6±0.19
Biochar 7	288±14.48	-17.3±1.39
Biochar 8	193±14.38	-27.7±2.26
Biochar 9	197±15.45	-18.4±0.15
Biochar 11	145±10.54	-75.2±3.5
Biochar 12	159±7.19	-27.9±1.76
Biochar 13	144±4.71	-30.5±1.88

All the surface area measurements were consistent with high mineral ash feedstock (Table 25). The highest surface area using a CO_2 adsorption method was detected in Biochar 5, and the lowest is measured in Biochar 3, which could be due to greater coverage of the surfaces with glycerol. The Biochar 3 had the highest positive zeta potential (highest negative charge on particles with an average diameter of 132 nm), followed by Biochar 4 and Biochar 5 (Table 17). The higher the zeta potential means higher probability that the biochar colloidal particles will not agglomerate in the ruminal fluid and will stabilise in the biofilms on the wall of the rumen (Sahle-Demessie and Tadesse, 2011).

Table 25. Surface area, micropore volume, colloidal particle size and zeta potential of the biochars tested in experiment 2 and 3.

Parameter	Biochar 3	Biochar 4	Biochar 5	Biochar 6
Surface area (m^2/g)	17.9	56.5	205.8	57.5
Micropore volume (cm^3/g)	0.008	0.02	0.08	0.02
Average particle size (nm)	132±4.46	154±8.51	219±14.45	281±60.59
Zeta potential (mV)	-85.6±9.07	-73.1±1.89	-50.4±2.65	-8.63±0.19

4.3.3 Inorganic chemical properties of biochars

Table 26 and 27 details the concentration of the non-Carbon and -N elements in the different biochars. Biochar 6 had the highest concentration of Na, Ca, Sr and Ba and Cl due to the added salt and lime. Biochar 4 had the highest content of Si, Fe, Mn, Mg and Al due to the addition of different minerals. Table 27 shows that there is a high content of Silica and K in the Biochar 5 sample and a high content of Ca and Mg in the Biochar 1.

Table 26. LA-ICP-MS of the Non-Carbon elements (mg/kg) in the biochars tested in experiment 1.

Biochars	Na	Mg	Al	Si	P	S	Cl	K	Ca	Fe	Mn	Cr	Ni	Cu	Zn	Sr	Cd	Ba
Biochar 3	4191	656	17338	91284	190	330	515	5232	16575	3056	132	73.4	6.52	5.79	11.9	196	0.46	180
Biochar 4	3915	4144	26512	111551	2091	581	2744	7962	21901	8485	571	35.8	18.6	9.37	61.8	515	0.51	428
Biochar 6	27388	2683	21435	97384	953	228	13162	3152	49235	1803	111	9.80	2.61	4.45	11.3	783	0.28	477
Biochar 7	568	1504	14045	33284	152	187	7639	624	48801	3932	234	29.5	6.62	8.07	28.6	451	0.36	44.1
Biochar 8	1341	1726	19532	56651	208	170	227	759	58301	3122	170	26.2	8.93	10.2	23.3	538	0.60	51.5
Biochar 9	941	1165	8378	24451	3167	56	260	433	56868	1075	85.4	20.8	4.96	8.40	14.3	519	0.43	28.5
Biochar 11	402	286	1019	308	61	211	68	1922	2148	327	14.8	12.1	2.09	2.85	2.80	17.5	0.30	5.86
Biochar 12	613	633	1596	4121	76	356	146	270	7265	757	23.2	17.9	3.30	5.24	10.3	35.3	0.33	8.19
Biochar 13	504	582	2635	5841	85	306	114	207	3481	770	30.1	20.8	3.93	6.34	10.1	28.0	0.40	11.0

Table 27. Ash analysis (%) of the biochars tested in experiment 1, measured by XRF.

Element Oxide	Biochar 1	Biochar 2	Biochar 5
Na_2O	2.28	1.84	3.84
MgO	4.50	3.93	1.54
Al_2O_3	15.64	17.15	6.14
SiO_2	63.69	58.86	73.59
P_2O_5	0.50	9.31	1.78
SO_3	0.31	<0.01	1.08
K_2O	1.72	1.71	5.33
CaO	2.60	1.00	2.15
TiO_2	0.78	0.64	0.32
V_2O_5	0.01	0.01	<0.01
Cr_2O_3	0.17	0.01	<0.01
Mn_3O_4	0.12	0.04	0.07
Fe_2O_3	5.53	5.67	2.80
NiO	0.16	<0.01	0.01
CuO	<0.01	<0.01	0.02
ZnO	<0.01	<0.01	0.05
SrO	<0.01	<0.01	<0.01
ZrO_2	0.01	<0.01	0.01
BaO	<0.01	<0.01	0.01
HfO_2	<0.01	<0.01	<0.01
PbO	<0.01	<0.01	<0.01
L.O.I.	ND	ND	ND
Ash content	4.07	14.05	17.25
TOTAL	98.02	100.17	98.74

Table 28 is a semi-quantitative analysis of the chemical elements detected in the ash including Pb and Cd. The EU limit for Pb and Cd in forage is 30 mg/kg and 1 mg/kg, respectively, and thus all biochars tested here pass the regulation (DIRECTIVE 2002/32/EC**)**, especially considering that they are added at either 1% or 5% of the total feed. The Biochar 6 biochar had a very high content of Na, Cl, Al, Si, Sr and Ba compared to the others. The Biochar 3 also had a high content of Mg, Al, Si, S and Fe due to the addition of clay and $FeSO_4$ in the formulation before pyrolysis. The As and Hg were not detected.

Table 28. Concentrations (mg/kg) of chemical elements of biochars tested in experiment 2 and 3.

Element	Biochar 3	Biochar 4	Biochar 6	Biochar 5
Na	3915	4191	27388	1341
Mg	4144	656	2683	1726
Al	26512	17338	21435	19532
Si	111551	91284	97384	56651
P	2091	190	953	208
S	581	330	228	170
Cl	2744	515	13162	227
K	7962	5232	3152	759
Ca	21901	16575	49235	58301
Fe	8485	3056	1803	3122
Mn	669	132	111	170
Cr	57	73	10	26
Ni	43	7	3	9
Cu	24	6	4	10
Zn	111	12	11	23
Sr	357	196	783	538
Cd	0.9	0.5	0.3	0.6
Ba	291	180	477	52
Pb	12	5	6	6
B	13	7	3	13

Raman spectroscopy was used to determine the nature of the carbon bonding in the biochar. Typically, two main bands are observed in Raman spectra. The G-band (graphite), which is centred at 1580 cm^{-1}, is observed for single crystal graphite, benzene and all aromatic rings. This peak arises from the in-plane vibrations of the sp2-bonded crystallite carbon. The other peak near 1357 cm^{-1} is denoted as the "disorder" peak (or D-band), which can be observed in polycrystalline graphite. This peak is attributed to in-plane vibrations of sp2-bonded carbon within structural defects. The G* peak at 1545 cm^{-1} represents aromatic semi-quadrant ring breathing for rings containing more than two fused aromatic rings. The greater the D/G ratio the more stable is the carbon structure and the greater the absorptivity of gases in the defect structure and the electrical conductivity and the potential for redox reactions to occur on the surface of the biochar(Chimowa *et al.*, 2017). Table 29 indicates that the D/G ratio is highest for the Biochar 4 biochar

Table 29. The ratio of D/G band intensity of the biochars tested in experiment 2 and 3.

Biochar	ID/IG
Biochar 3	2.9
Biochar 4	3.2
Biochar 5	2.8
Biochar 6	2.7

4.3.4 The ICP-MS and LC-OCD of soluble metals non-metals and organic compounds

Table 30 shows that the Biochar 6 and Biochar 9 biochar samples had the highest concentration of soluble micro-nutrients but also the highest concentration of heavy metals. The Biochar 4 had a high concentration of soluble S, K, Mg and some heavy metals, such as Co and Zn.

Table 31 shows that the highest dissolved organic carbon and low molecular weight neutrals was measured in Biochar 6, Biochar 11, and Biochar 3. This is due to the addition of the glycerol. Biochar 6 also had a very high concentration of humic-like-substances and building blocks (polyphenols/ polyaromatic acids) and low molecular weight acids. This is related to the addition of molasses, as well as using a low temperature biochar.

Table 30. Elemental ICP-MS of liquid after solubilisation of the biochars tested in experiment 1.

	Biochar 1	Biochar 3	Biochar 4	Biochar 5	Biochar 6	Biochar 7	Biochar 8	Biochar 9	Biochar 11	Biochar 12	Biochar 13
Al (mg/l)	0.28	2.67	0.61	0.41	6.15	0.03	0.01	0.02	1.56	1.1	0.21
B (mg/l)	0.02	0.13	0.84	0.33	0.22	0.48	0.34	0.38	0.06	0.05	0.13
Ca (mg/l)	12.6	2.2	778	30.4	1790	1463	202	290	0.31	14.1	13.8
Fe (mg/l)	0	0.96	0.31	1.16	3.3	0.04	0	0	0.95	0	0
K (mg/l)	33.4	343	1209	364	289	22.3	8.17	11.2	182	28.2	6.57
Mg (mg/l)	9.36	0	118	0.9	93.1	35.8	17.2	23.4	0	4.13	2.63
Na (mg/l)	20.3	9.1	102	117	8313	23.5	21	20.14	21.6	27.2	15
P (mg/l)	0.43	2.94	0	33.9	10.7	0.45	0.14	281	2.9	0.36	0.07
S (mg/l)	3.26	18.9	453	22.6	57.9	27.8	31.3	25.2	9.96	15.4	2.73
Si (mg/l)	3.95	3.67	34.5	5.05	14.9	13.6	9.28	27.1	4.66	2.17	3.73
Sr (mg/l)	0.13	0.11	10.3	0.32	18.9	15.3	2.69	4.7	0.03	0.19	0.11
Ba (ug/l)	41.6	30.4	250	19	2640	324	74.1	11.2	12.6	15.7	2.82
Cd (ug/l)	0.07	0.08	5.16	0.21	2.28	0.31	0.11	0.14	0.19	0.04	0.14
Co (ug/l)	0	1.49	540	2.3	29.1	64.6	0.65	16.6	0.43	0	0
Cr (ug/l)	0	5.16	0.13	9.19	30.8	0.4	0	1.52	7.54	0.43	0.98
Cu (ug/l)	0	9.63	4.15	25.4	24.2	0.48	0.19	0.04	18.3	0	0
Li (ug/l)	3.92	3.45	99.3	6.15	18.1	44.2	12.9	34.4	1.17	2.15	1.57
Mn (ug/l)	38.2	72.8	31.5	117	1121	5190	237	768	36.8	10.7	3.19
Mo (ug/l)	3.51	4.56	0.20	2.26	41.9	1.69	3.48	1.02	1.97	4.54	1.94
Ni (ug/l)	0.32	12	67	2.37	63.7	8.07	0.37	3.92	14.4	0.06	0.3
Pb (ug/l)	0.07	0.64	40	0.48	17.8	0.14	0.04	0.02	1.34	0.03	0.05
Ti (ug/l)	1.64	4.84	380.6	9.66	48.2	0	0	0	4.83	0	1.46
Zn (ug/l)	1.92	29.1	960	34.7	73.9	230	6.07	10.3	31.5	2.13	2.33
Zr (ug/l)	8.07	6.83	0.18	6.42	48.3	2.63	3.5	2.99	9.27	11.2	4.01
Se (ug/l)	4.56	5.45	0	3.3	3.16	0	2.39	3.71	0	1.3	4.19
Cl (mg/k)	15	82	0	351	15271	2587	22	102	16	24	8.7

Table 31. Water-soluble organic compounds of the biochars tested in experiment 1.

Compound (mg/g)	Biochar 1	Biochar 3	Biochar 4	Biochar 5	Biochar 6	Biochar 7	Biochar 8	Biochar 9	Biochar 11	Biochar 12	Biochar 13
DOC	0.13	34.0	0.09	2.44	70.4	2.17	4.10	2.47	40.1	0.038	0.16
HOC	0.035	6.14	0.04	1.03	14.3	0.40	0.75	0.43	15.0	0.008	0.01
CDOC	0.096	27.8	0.05	1.40	56.0	1.75	3.40	2.04	26.0	0.03	0.15
Bio-polymers	n.q.	0.12	n.q.	0.15	4.40	0.01	0.04	0.02	0.14	n.q.	0.02
Humics	0.015	1.16	n.q.	0.85	9.16	0.27	0.50	0.40	0.65	0.008	0.01
Building blocks	0.008	0.81	0.04	0.16	18.6	0.60	1.70	0.90	1.05	0.005	n.q.
LMW neutrals	0.06	25.58	0.004	0.24	20.2	0.13	0.24	0.15	23.8	0.015	0.11
LMW acids	0.01	0.10	n.q.	0.02	3.60	0.70	0.94	0.57	0.30	n.q.	n.q.
Aromaticity (L/(mg*m))	1.53	2.16	n.q.	5.33	1.32	1.55	2.21	1.86	3.87	2.34	2.29

DOC= Dissolved Organic Carbon, HOC= hydrophobic OC, CDOC= Hydrophilic OC, n.q.: no quantified

Biochar 6 and Biochar 4 biochar samples had the highest concentration of soluble micro-nutrients, but also the highest concentration of heavy metals, although these were within safe levels (Table 32). Given that these 2 biochars had the lowest methane production when fermented in the Rusitec, it is possible that some of these cations and anions play a role in either altering abundance of specific micro-organisms or has some effect on abiotic redox reactions. Biochar 3 had a high concentration of soluble S, K, Mg and some heavy metals, such as Co and Zn.

Table 32. ICP-MS of liquid after solubilisation of the biochars tested in experiment 2 and 3.

Element	Biochar 3	Biochar 4	Biochar 5	Biochar 6
Al	2.67	0.61	0.41	6.15
B	0.13	0.84	0.33	0.22
Ca	2.2	778	30.4	1790
Fe	0.96	0.31	1.16	3.3
K	343	1209	364	289
Mg	0	118	0.9	93.1
Na	9.1	102	117	8313
P	2.94	0	33.9	10.7
S	18.9	453	22.6	57.9
Si	3.67	34.5	5.05	14.9
Sr	0.11	10.3	0.32	18.9
Ba	30.4	250	19	2640
Cd	0.08	5.16	0.21	2.28
Co	1.49	540	2.3	29.1
Cr	5.16	0.13	9.19	30.8
Cu	9.63	4.15	25.4	24.2
Li	3.45	99.3	6.15	18.1
Mn	72.8	31.5	117	1121
Mo	4.56	0.2	2.26	41.9
Ni	12	67	2.37	63.7
Pb	0.64	40	0.48	17.8
Ti	4.84	380.6	9.66	48.2
Zn	29.1	960	34.7	73.9
Zr	6.83	0.18	6.42	48.3
Se	5.45	0	3.3	3.16
Cl	82	0	351	15271

The highest dissolved organic carbon (DOC) and low molecular weight neutrals was measured in the Biochar 6 and Biochar 3 (Table 33). The low concentration of DOC in the Biochar 4 indicates that either the organics were not water soluble, or they were bound very tightly to the biochar and the minerals. The Biochar 6 also had a very high concentration of humic-like-substances, building blocks (polyphenols/ polyaromatic acids) and low molecular weight acids. This is related to the addition of molasses as well as the use of a low temperature biochar. It is possible that the high reduction in methane could have been due in part to the high electron acceptor capacity of the humics and polyphenols (Avila-Stagno *et al.*, 2014, Hsueh *et al.*, 2019).

Table 33. Summary of the results from the analysis of the water-soluble organic compounds: DOC - Dissolved Organic Carbon, HOC - hydrophobic OC, CDOC - Hydrophilic OC of the biochars tested in experiment 2 and 3.

Compound (mg/g)	Biochar 3	Biochar 4	Biochar 5	Biochar 6
DOC	34.0	0.09	2.44	70.4
HOC	6.14	0.04	1.03	14.3
CDOC	27.8	0.05	1.40	56.0
Bio-polymers	0.12	n.q.	0.15	4.40
Humics	1.16	n.q.	0.85	9.16
Building blocks	0.81	n.q.	0.16	18.6
LMW neutrals	25.6	0.04	0.24	20.2
LMW acids	0.10	0.004	0.02	3.60
Aromaticity (L/(mg*m))	2.16	n.q.	5.33	1.32

4.3.5 X-ray Proton Spectroscopy

The XPS analysis of the C and N functional groups and the major elements on the surface of crushed biochar are shown in Table 34. The Biochar 3 had a much smaller concentration of aromatic and aliphatic carbon and a greater content of minerals that had a high concentration of oxygen than the other samples. The Biochar 3 and Biochar 6 had a similar and high concentration of alcohol esters and ethers (C-O and C-OC bonds). The Biochar 6 had a high concentration of C=O associated with ketones that could have been derived from the acid activation of the biochar and/or from the addition of glycerol. The Biochar 5 had the highest concentration of carboxyl functional groups, indicating that it should have the greatest ability to hold cations.

The Biochar 4 was the only biochar to have detectable N functional groups associated with amine and nitrates. The amines come from the glycerol and the nitrates from the added potassium nitrate. The nitrates and possibly the amines can act as electron acceptors and thus are one of the potential mechanisms that led to the reduction in methane. The XPS spectra indicated that there are both Fe^{2+} and Fe^{3+} oxidation states detected on the surface of Biochar 3 but not on the other biochars. Where 2 oxidation states exist the so-called redox wheel process can occur whereby N, S, and P become more available for transport from the rumen into the animal (Li *et al.*, 2012). Biochar 6 had the greatest concentration of Na and Cl on the surface while Biochar 3 had the highest concentration of all other elements on the surface. The Na in the rumen has been associated with an increase in acetogens, which can produce acetate from hydrogen and CO_2 (Muller, 2003).

Table 34. The XPS analysis of the C and N functional groups and the major elements on the surface of the biochars tested in experiment 2 and 3.

Name	Functional groups	Peak BE	Biochar 3 (%)	Biochar 4 (%)	Biochar 5 (%)	Biochar 6 (%)
C1s A	C-C/C-H	285	76.21	10.2	53.5	60.7
C1s B	C-O/C-OC	286	3.46	13.8	17.9	8.17
C1s C	C=O	288	2.74	3.47	3.25	3.96
C1s D	O=C-O/Carboxylic	289	1.93	1.75	2.50	1.29
N1s A	NH_4/NH_2	399	0.87	n.d	0.2	0.62
N1s B	N-C-COOH /Pyridone	401	n.d	n.d	1.06	n.d
N1s C	NH_3+/amines	402	n.d	0.26	n.d	n.d
N1s D	Nitrates/nitrites	409	n.d	1.06	n.d	n.d
O1s A		531	10.32	10.9	11.9	11.7
O1s B		533	2.06	33.9	7.47	7.15
Ca2p3 A		348	1.43	2.98	0.12	2.01
Mg1s		1304	n.d	3.56	0.30	n.d
Si2p		104	0.66	4.70	0.21	1.11
Cl2p3 A		200	n.d	1.06	n.d	1.87
Cl2p3 B		198	n.d	n.d	0.19	0.68
S2p3 A		165	n.d	0.81	n.d	n.d
S2p3 B		171	n.d	1.96	n.d	n.d
Na1s A		1072	0.16	0.28	0.42	0.60
Al2p		74.8	n.d	1.73	n.d	n.d
P2p		134	n.d	0.97	0.28	0.16
K2p3 A		292	1	2.4	0.62	n.d
Fe2p3 A		713	n.d	1.97	n.d	n.d
Fe2p3 B		717	n.d	0.91	n.d	n.d
Fe2p3 C		721	n.d	0.66	n.d	n.d

n.d.: no detected

4.3.6 GC-MS identification of the solvent extracted organic molecules

Complete results are shown in Appendix 8.2. All the biochars had a range of methyl esters, alkanes, and hexadecenoic acids. There are considerable differences between the biochars. Biochar 5 was the only biochar to have benzoic acid, benzaldehyde, and vanillin. There was lauric acid (Dodecanoic acid) found in Biochar 5 and Biochar 4 and myristic acid (Tetradecanoic acid) found in all biochars except Biochar 3, which are reported to be antibacterial. These acids have been found to effect microbial populations in the rumen (Francisco *et al.*, 2019). Linoleic acid, which can be a defence mechanism against the toxic effects of dietary PUFA was found in Biochar 6 and Biochar 3. These acids are involved in rumen FA biohydrogenation (progressive H saturation of fats and oils), a pathway that competes for methane in the rumen (Patra *et al.*, 2017).

4.3.7 Scanning Electron Microscopy analyses of the biochars

A fragment of biochar that has a high concentration of nanoparticles rich in P, K, Na, Cl and a small amount of Fe and Mg is shown in Fig. 9 (top). The bottom image in Fig. 9 shows the porous nature of the mixed feedstock and the significant concentration of a range of micronutrients. These mineralised surfaces in the pores are sites where specific micro-organisms can multiply.

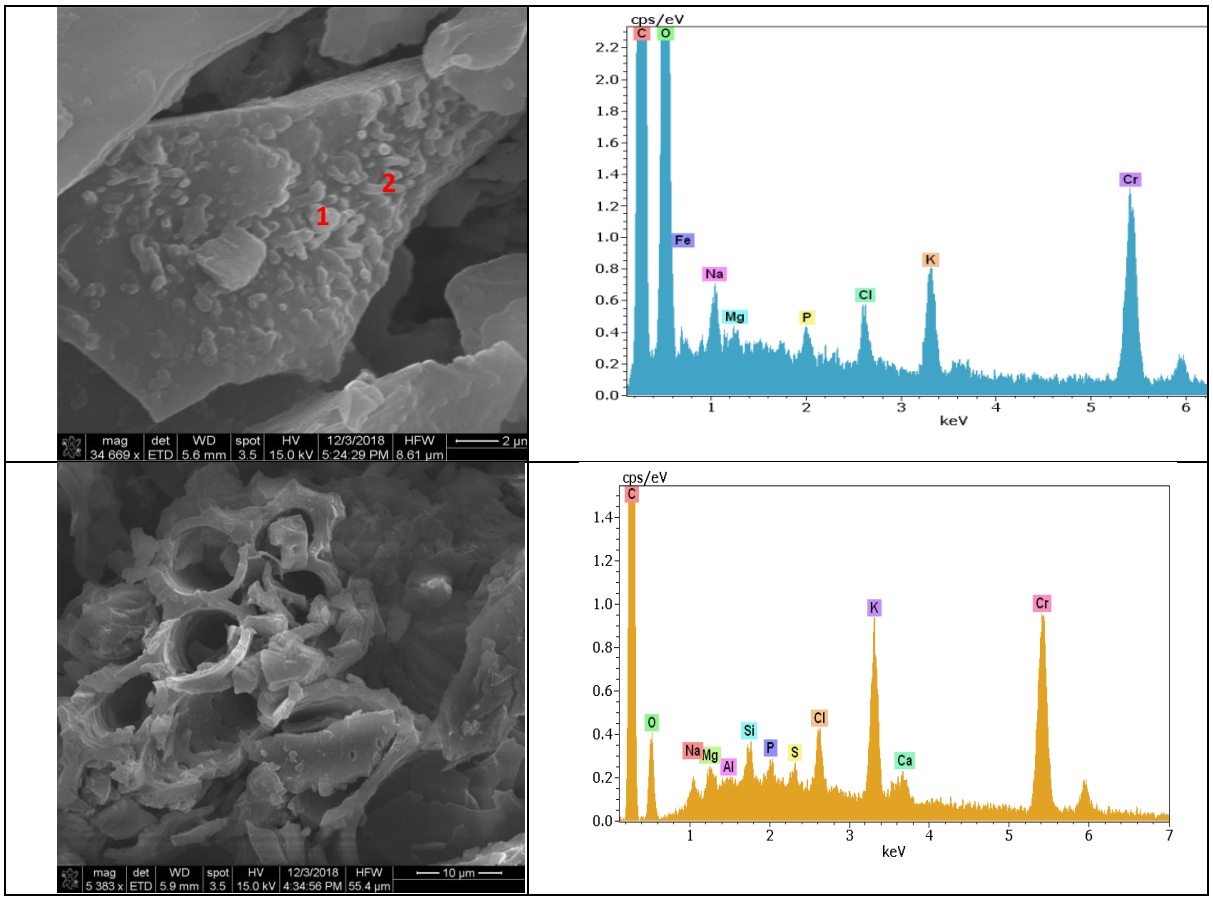


Figure 9. Secondary electron image with elemental analysis using Energy Dispersive X-Ray Spectroscopy (EDS or EDX) of the surface of Biochar 5.

The image in Fig. 10 shows that the pores of the biochar have been filled with Ca, Mg and Na, while Fig. 11 shows that the surface of the Biochar 6 biochar has a high content of Al and Si micron and sub-micron particles (probably clay and sand from the biomass residues that have been taken from the field) as well as salt and lime.

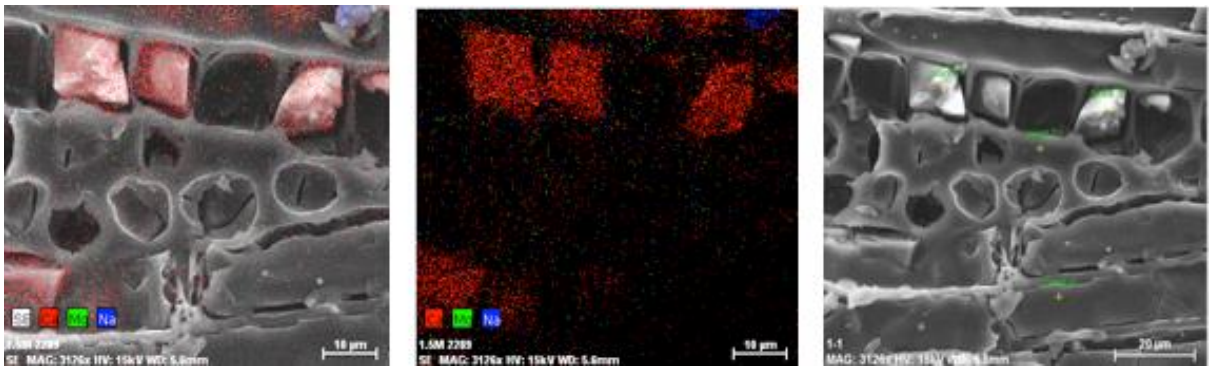


Figure 10. Secondary electron image of the pores of Biochar 6 filled with minerals with Ca (probably lime) Na (salt) and a very small amount of Mg.

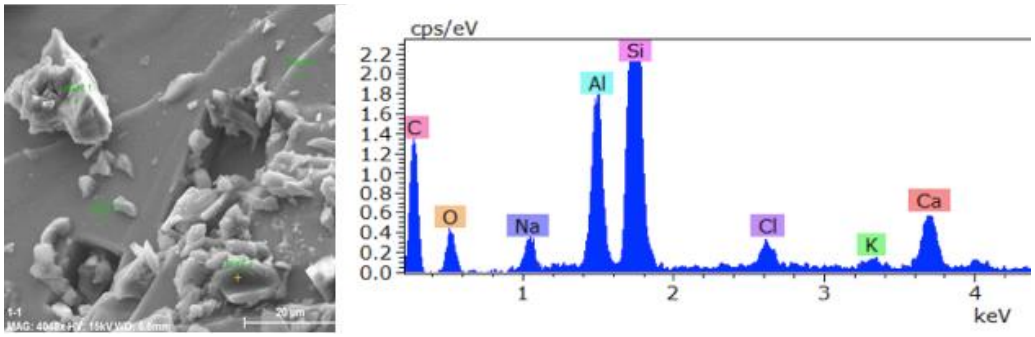


Figure 11. Secondary electron image of the surface of Biochar 6 filled with minerals with Ca, Na, and a very small amount of Mg

The inclusion of a range of minerals in the pores of both Biochar 3 and Biochar 4 samples can be observed in Fig. 12 and 13. Biochar 4 has a much more diverse and higher concentrations of both macro and micro-minerals.

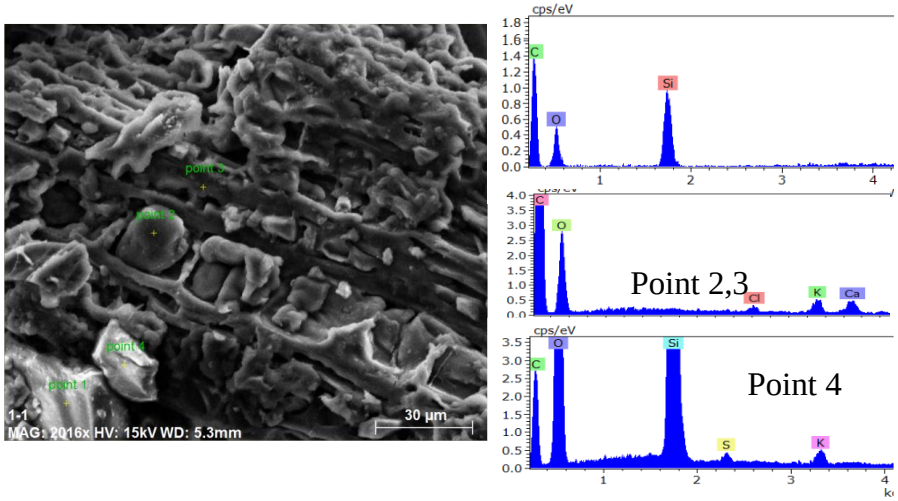


Figure 12. Secondary electron image of the surface of wood Biochar 3 filled with a range of minerals in the pores. EDS analysis is carried out at 4 points

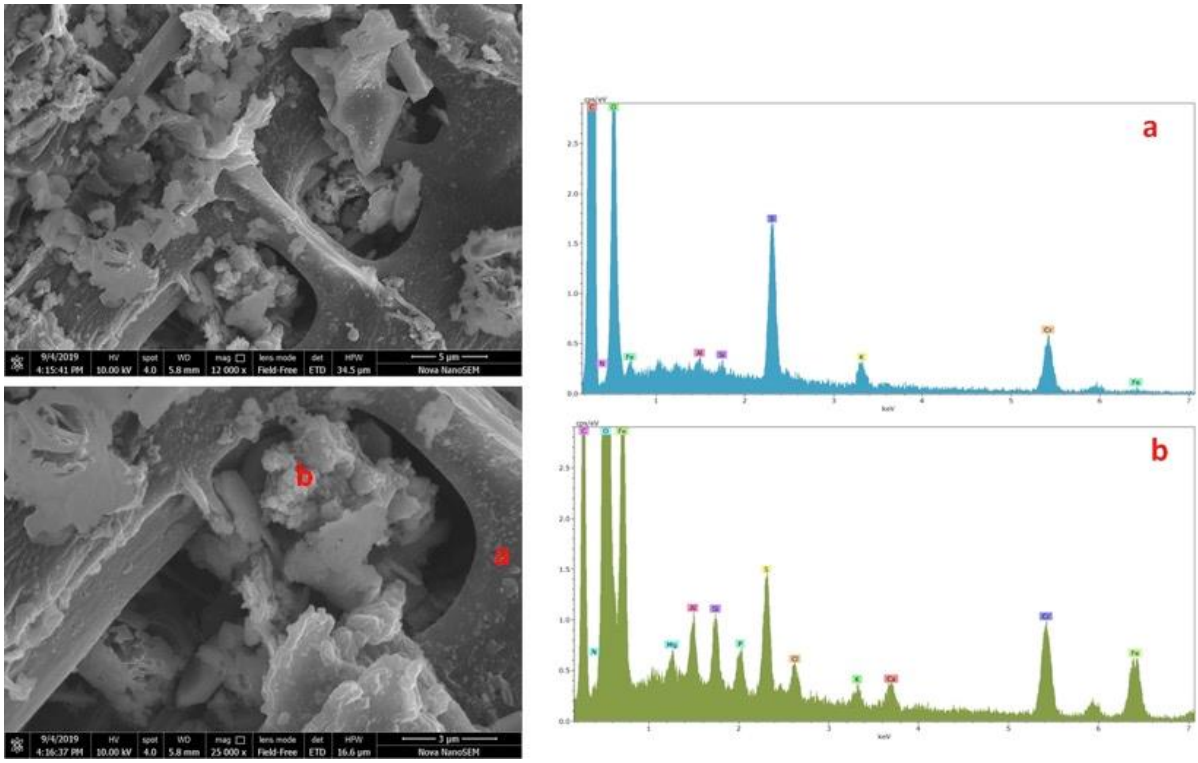


Figure 13. Secondary electron image of the surface of wood Biochar 4 filled with a range of minerals in the pores.

4.3.8 Characterisation of biochars used in the animal trials (new batches)

There were significant differences between the 2 biochars in terms of the basic properties. Biochar 6 had a higher pH, acid neutralising capacity and EC and a lower moisture, total organic carbon surface area and ash content than Biochar 4.2 NT6 (table 35). Biochar 6 had a much greater content of higher Na Ca Zn Cu Co lower content of Fe and Al (table 36). PCB and PAH were not detected in either biochar.

Table 35. Basic characteristics of the biochars produced for the animal trials

Parameter	Biochar 6	Biochar 4.2 NT6
Bulk Density (kg/L)	0.82	0.76
Moisture Content (%)	6.7	25.5
Volatile Matter (%)	47.4	39.8
Ash Content (% ash)	52.6	60.2
pH	9.73	4.91
Electrical Conductivity (dS/m)	50.27	10.22
Sulfur (%S)	1.08	0.32
Hydrogen (%)	1.50	1.71
Oxygen (%)	24.16	12.85
Organic Carbon (%)	13.7	24.2
Carbon (%)	20.5	23.8
Nitrogen (%)	0.17	1.04
Acid Neutralising Capacity (% $CaCO_3$)	24.34	0.24
Surface Area		
Single Point Surface Area P/PO (m^2/g)	0.1359	6.4509
BET Surface Area (m^2/g)	0.1427	6.5050
Langmuir Surface Area (m^2/g)	0.1884	8.5971

Table 36. Metal and non-metal content the biochars produced for the animal trials.

	Biochar 6	Biochar 4.2 NT6
%		
Ca	17.03	1.16
Mg	0.17	0.24
K	0.29	2.89
Na	13.29	0.54
S	1.08	0.32
P	0.02	0.11
(mg/kg)		
Zn	167	31
Mn	57	98
Fe	849	7,080
Cu	253	6
B	<5	8.3
Si	528	698
Al	615	9,496
Mo	<1	<1
Co	33.9	2.40
Se	7.59	<1
Cd	0.5	<0.5
Pb	<1	5.2
As	<2	2.48
Cr	3.2	6.7
Ni	<1	5.0
Hg	<0.1	<0.1
Ag	<1	<1

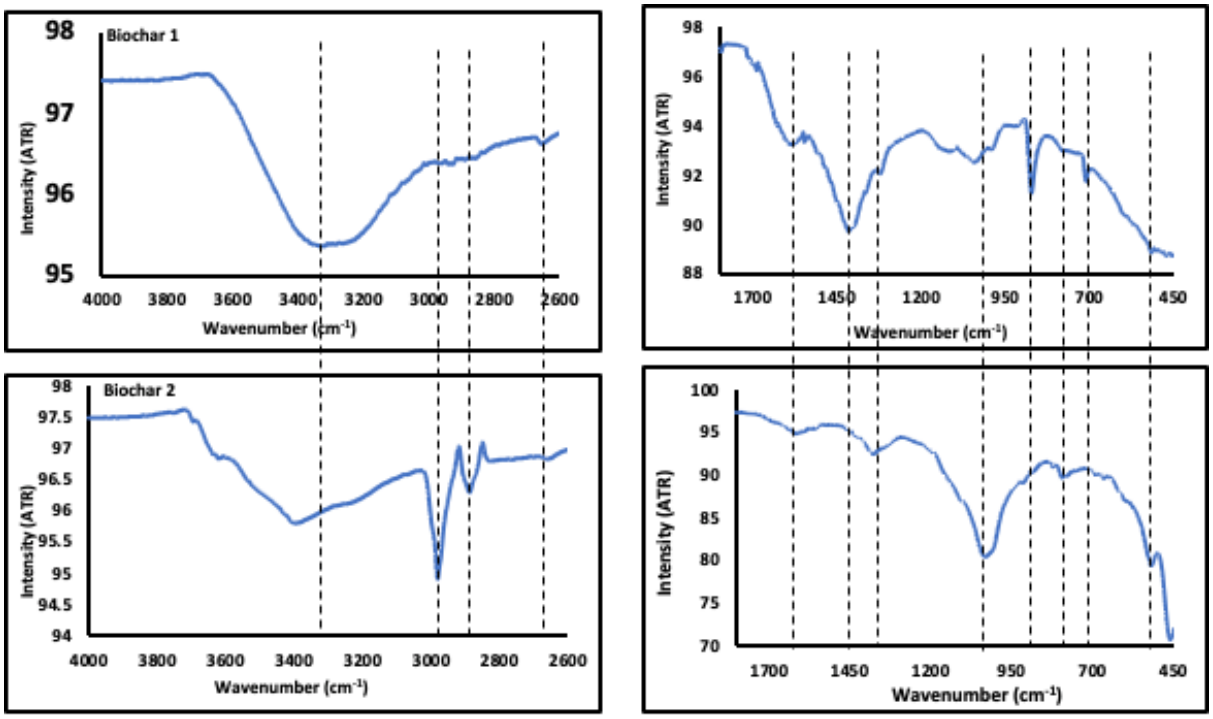


Figure 14. FTIR spectrum of the biochars produced for the animal trials.

Two areas are shown in the FTIR spectra (Fig. 14): the peaks in the range between 4000 and 2600 cm-1 and the peaks in the range between 1800 and 450 cm-1. These function groups determine the ability of the biochar to bind with different nutrients when ingested by the animal. The broad peak in the range between at 3400 cm-1 and 3200 cm-1 can be assigned to O-H (alcohol or phenol) and aromatic C-H stretch (alkenes); This broad peak, which is less intense in Biochar 4.2 NT6 and appeared as a sharp single peak in the Biochar 4.2 NT6 (at about 3390 cm-1). The two sharp peaks at about 3000 cm-1, attributed to either C-H stretch in alkanes or O-H in carboxylic acids, and 2800 cm-1 were only found in Biochar 4.2 NT6. The intense peak at about 1415 cm-1 in Biochar 6 can be attributed to the C-H in alkanes, aldehydes and ketones; this peak can also either for C-O-H bending in carboxylic acids (consistent with XPS analysis). The peak at about 1020 cm-1 (and 1120 cm-1 in Biochar 6) could be for different organic and inorganic compounds, including phosphate, silicate, Al-O and/or C-O with a higher intensity of this peak was identified in Biochar 4.2 NT6. Small peaks in Biochar 4.2 NT6 (in the wavenumbers ranged between 800-600 cm-1) could be a signature of Al-O and Si-O.

Table 37. XPS analysis of the surface of the biochars produced for the animal trials.

Name	Functional groups	Peak BE	Biochar 6 (%)	Biochar 4.2 NT6 (%)
C1s A	C-C/C-H	284.8	34.01	14.24
C1s B	C-O/C-OC	286.0	10.59	3.6
C1s C	C=O	287.8	2.38	1.55
C1s D	O=C-O/Carboxylic	289.2	5.65	1.09
O1s A		532.02	13.94	54.55
O1s B		533.34	9.05	
N1s A	N-C-COOH /Pyridone	400.1	0.65	0.26
N1s B	Nitrate	407.69		1.97
N1s C	Nitrite	403.88		0.22
K2p3 A		293.73	0.63	2.77
Ca2p3 A		347.92	5.53	0.95
S2p3 A		163.97	0.54	
S2p3 B		162.05	0.14	
S2p3 C	Thiosulphate	168.84	0.16	
Cl2p3 A		199.42	10.55	1.35
Na1s		1072.41	6.17	0.58
Fe2p3 A	Fe2(SO4)2	712.77		0.43
Fe2p3 B	Fe3+	715.31		0.13
Si2p A		103.4		9.95
Al2s A		120		4.27
Mg1s A		1304.42		2.08

XPS analysis of the surface (Table 37) indicated that the concentration of total surface C functionalities was higher in Biochar 6 (52.63 %) than Biochar 4.2 NT6 (20.48 %). The relative portion of C-O and carboxylic functional group were greater in sample 1, while the ratio of surface C-C/C-H, C=O were higher in sample 2. Sample 2 showed a higher total surface N functionality with nitrate and nitrite only detected in sample 2. Similarly, and Fe functional groups, including Fe2(SO4)2 and Fe3+, Mg, Al and Si functionalities were only detected in sample 2. No S functionalities was found on the surface of sample 2; a significantly higher surface Na functional group was found in sample 1 (6.17 %).

Table 38. Analysis of soluble organic compounds by Liquid Chromatography the biochars produced for the animal trials; DOC: Dissolved organic carbon, HOC: Hydrophobic organic carbon, CDOC: Chromatographable organic carbon .

Metabolites (mg/g)	Biochar 6	Biochar 4.2 NT6
DOC	15.41	0.08
HOC	3.6	0.024
CDOC	11.82	0.054
Bio-polymers	0.11	0.001
Humics	2.6	0.013
Building blocks (polyphenols)	1.37	0.005
LMW neutrals	7.73	0.034
LMW acids	n.q.	n.q.

Biochar 6 contained a significantly higher soluble DOC content than Biochar 4.2 NT6 and the majority of the organic compounds were low molecular weight neutrals and humic like substances (Table 38). These can be involved in redox chemistry in an acid environment (Zhou *et al.*, 2014).

Table 39. Zeta potential and particle size of the biochars produced for the animal trials

Sample	Particle size (nm)	Zeta potential (mV)
Biochar 6	389.5	-12.5
Biochar 4.2 NT6	156.6	-3.39

The Biochar 6 had a higher negative zeta potential (Table 39) than Biochar 4.2 NT6 but both were significant less than the zeta potential of most methanogens (Ahammad *et al.*, 2013).

Table 40. Specific capacitance of the biochars produced for the animal trials

	Specific capacitance (F/g)
Biochar 6	0.317
Biochar 4.2 NT6	0.416

Biochar 4.2 NT6 showed a slightly higher specific capacitance than Biochar 6. However, both biochars might have the ability to donate or accept electrons during fermentation in the rumen.

4.4 Ex-post cost benefit analysis

Ex-post cost benefit analysis of the 2 biochars tested as feed supplements for cattle under extensive grazing conditions was conducted using the results from *in vivo* experiments 2 & 3 (Table 42). The benefits selected for the analysis were enteric methane abatement and animal productivity

improvements and no other benefits were considered. A negative return to investment of -0.066$/animal/day and -2.36$/animal/day for Biochar 6 and Biochar 4.2 NT6 respectively (only considering the cost of biochar) was obtained under the conditions tested, as no significant methane reduction or productivity improvement were observed when biochars were fed to grazing cattle for 60 days.

Table 42. Ex-post cost benefit analysis of the use of Biochar 6 and Biochar 4.2 NT6 as feed supplement for grazing cattle.

	Biochar 6	Biochar 4.2 NT6
Supplement cost (per kg supplement)	$1	$35.75
Supplement administration rate (g supplement/head/day)	66	66
Supplement cost (animal/ day)	$0.066	$2.36
Methane reduction	0	0
Change in productivity	0	0
Return to investment (animal/day)	$-0.066	$-2.36

4.5 Review of engagement and communications activities

The project completed a wide range of communication activities targeting red meat producers, particularly grass-fed systems, to communicate about the project targets and progress.

- Biochar Network (WA). Presenter Phil Vercoe: "*Fit-for-purpose biochar for livestock"* (April 1, 2019)
- Research team visit to Doug Pow's property (April 17, 2019)
- Visit to Doug Pow's property in Manjimup with MLA (Nov 8, 1019)
- ANZ Biochar Industry Group (ANZBIG), Biochar for Animal Health & Wealth seminar series. 26th November 2020. Presenters: Stephen Joseph & Gonzalo Martinez-Fernandez
- 33rd Biennial Conference of the Australian Association of Animal Sciences. 1-3 Feb 2021, Fremantle (WA). Poster: *"Selected biochars can reduce methane production in vitro"*. Presenter Zoey Durmic
- Biochar project progress included in CSIRO annual report to NABRC meeting (2020 & 2021).
- Beef week 2021 (Rockhampton). MLA display included information about progress of Biochar project.
- Lansdown Beef Research Field Day, September 2021 (Lansdown Research Station, QLD): communicated about the project targets and progress.
- Radio interview about progress of the project completed during Lansdown Beef Research Field Day, Sep 2021. ABC Southern Queensland (Country hour). Gonzalo Martinez-Fernandez
- 34th Australian Association of Animal Sciences Conference. 5-7 July 2022. Oral presentation submitted: "*Biochar supplementation for beef cattle: methane emissions and grazing applications".* Presenter: Gonzalo Martinez-Fernandez

4.6 Development for biochar methodology and engagement with DoEE

The Emissions Reduction Fund (ERF) incentivises Australian businesses to cut the amount of greenhouse gases they create and to undertake activities that store carbon. The Clean Energy Regulator develops methodologies in consultation with industry, potential end-users, scientists and technical experts and the Emissions Reduction Assurance Committee. The Committee assesses

whether methods comply with the offsets integrity standards set out in the legislation (Emissions Reduction Fund | Department of Industry, Science, Energy and Resources).

Based on the results generated in the grazing trials, no methodology could be developed to use biochar for reducing methane emissions in cattle production systems at this stage (with the current biochars and production systems tested). No significant methane reduction or productivity improvements were observed between control and biochar treated animals under grazing conditions with the two candidates studied, despite some promising results *in vivo* under a tightly controlled feeding regime.

5. Conclusion

We have custom-designed biochars with properties that modified rumen fermentation parameters in both batch and continuous culture systems *in vitro*. Four of the biochars showed a persistent effect on methane and other fermentation parameters when tested in continuous culture systems. These 4 biochars also reduced methane (8.8-12.9 % reduction) without affecting productivity and caused a change in the ruminal microbial populations when compared to the control animal under controlled feeding conditions and methane measurements were taken in over 48 hours in respiration chambers. The characterisation of the biochars indicated that the parent material and post pyrolysis modifications (such as the addition of nitrates) might be responsible for effects on rumen fermentation we measured. These effects could not be detected when two of the most promising biochars were tested in grazing animals. Based on our results with the biochars that we tested in grazing trials, and only considering the cost of the supplement in relation to the methane reduction and productivity measurement, it would not be cost effective to feed biochar as a supplement to reduce methane emissions in cattle. At this stage it would not be possible to develop a new Emissions reduction fund (ERF) methodology by The Clean Energy Regulator under current guidelines. Key findings

- The 14 biochars tested showed a wide variability on methane inhibition, from no effect on methane production to up to a 41% inhibition *in vitro* and negative impact on rumen fermentation. Not all the biochars were able to significantly decrease enteric methane and their effects depended on different characteristics such as the biomass used, pre- and post-pyrolysis manipulation, other inherent compounds and dietary inclusion rate.
- Two biochar types – a custom-made biochar Biochar 4.2 NT6 and a commercial biochar Biochar 6 showed the strongest potential to inhibit methane in ruminants and were tested in vivo. Their anti-methanogenic effects were likely to be due to different properties: the custom-made biochar Biochar 4.2 NT6 contained nitrates, which are an indirect inhibitor of methanogenesis, was acidic, substrate based on *Eucalyptus globulus*, straw, bentonite, zeolite and pyrolysis temperature of 600°C, while the commercial biochar Biochar 6was alkaline, parent substrate based on *Acacia cambagei* and pyrolysis temperature of 450°C.
- The mode of action of biochars is likely to be through indirect inhibition of methanogenesis rather than direct action on the methanogens themselves.
- The two biochars and doses tested reduced CH_4 emissions (8.8-12.9 % reduction) in cattle under controlled feeding conditions without any detrimental effect on rumen fermentation or feed intake. A dose response effect on methane reduction was only observed in Biochar 4.2 NT6.
- Under grazing, no significant difference on enteric CH_4 emissions or productivity could be detected when biochars were supplemented to cattle over 60 d.

5.1 Benefits to industry

This project focussed on enteric methane mitigation and productivity in cattle but could not identify any associated benefit to the red meat industry (particularly in grazing systems) with the biochars and doses tested. Under grazing conditions no significant differences on enteric CH_4 emissions or productivity were detected when cattle were supplemented with the biochars. Further research will be required to identify a fit-for-purpose biochar suitable for grazing systems. The successful candidate would require a much greater methane reduction under controlled feeding conditions (3-4 times or more than the reduction achieved in the current project) to be suitable for use as an anti-methanogenic supplement under grazing conditions, this is to compensate for the variables in grazing systems that could dilute the effect detected in respiration-chambers.

6. Future research and recommendations

Some of the biochars initially tested were able to decrease methane emissions under controlled feeding conditions in cattle, but results indicated that both biochars did not have sufficient methane reduction capability (8%–13%) or productivity benefits to warrant engaging a third party to develop a business case for the further development of palatable biochar supplements for commercial application. The results do demonstrate that it is possible to custom-design biochars to influence the end products of fermentation in the rumen. Further research could identify a fit-for-purpose biochar with much greater anti-methanogenic properties (3-4 times or more than current project, to compensate for the variables in grazing systems that could dilute the effect detected in respiration-chambers) to be commercially-viable for Australian grazing systems. The biochars assessed in this project did not offer methane abatement levels that would allow it to be classified as anti-methanogenic for commercial purposes. Our results do advance the field of biochar use as a supplement for ruminants and could form the basis for more strategic manipulation of biochar to have greater effects in the rumen.

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8. Appendix

8.1 List of biochars tested in the project

Treatment name	Aged	Biomass source	Temp °C	Post-pyrolysis Alterations
Biochar 1	Fresh	Wood *Eucalyptus species* (mixed, unspecified)	600	Added bentonite, coated with glycerol (1%)
Biochar 2	Fresh	Wood *Eucalyptus species* (mixed, unspecified)	600	Added bentonite, Acidified with 6% phosphoric acid (15.2 M)
Biochar 3	Fresh	Zeochar + bark *(Eucalyptus pilularis)*	600	Acidified 6% HCl (11 M). Coated with glycerol (20%)
Biochar 4	Fresh	2 part straws; 2 part hardwood, 1 part wheat straw ash *Eucalyptus species* ('ironbark', *E. globulus*)	600	KNO_3 (3.33%)
Biochar 5	Fresh	Untreated organic green waste woodchip *Eucalyptus species* (mixed, unspecified) and *Melaleuca alternifolia*	350	-
Biochar 6	Fresh	Wood (*Acacia cambagei*)	450	Mixed with 31.5% salt, 30% $CaCO_3$ and 8.5% molasses
Biochar 7	Fresh	Wood (*Acacia cambagei*)	450	Acidified with 6% HCl (11 M)
Biochar 8	Fresh	Wood (*Acacia cambagei*)	450	Mixed with 8.5% molasses
Biochar 9	Fresh	Wood (*Acacia cambagei*)	450	Acidified with 6% phosphoric acid (15.2 M)
Biochar 10	Fresh	Wood (*Acacia cambagei*)	450	-
Biochar 11	Aged	Jarrah wood, (*Eucalyptus marginate*)	600	glycerol (1%)
Biochar 12	Fresh	Jarrah wood, (*Eucalyptus marginate*)	600 C	glycerol (1%),
Biochar 13	Aged	Jarrah wood, (*Eucalyptus marginate*)	600 C	-
Biochar 14	Fresh	Jarrah wood, (*Eucalyptus marginate*)	600 C	-

8.2 Organic compounds identified by GC-MS in the four selected biochars.

Organic compound (% area from the chromatogram)	Biochar 3	Biochar 4	Biochar 5	Biochar 6
10-Undecenoic acid, methyl ester	0.56	0	0	0
11-Octadecenoic acid, methyl ester	2.08	0	0	0
13-Docosenamide, (Z)-	1.04	22.16	3.1	0
1-Decanol, 2-hexyl-	0	1.65	0	0.53
1H-Benzocyclohepten-7-ol, 2,3,4,4a,5,6,7,8-octahydro-1,1,4a,7-tetramethyl-, cis-	0.77	0	0	0
1-Heptadecanol	0	0.53	0	0
1-Hexanone, 1-phenyl-	0	0	0	0.19
1-Phenyl-1-butene	0	0	0	0.32
2(1H)-Naphthalenone, octahydro-, trans-	0.41	0	0	0
2,4,6(1H,3H,5H)-Pyrimidinetrione, 5,5-diethyl-1-methyl-	0	0	1.66	0
2,4-Decadienal, (E,E)-	0	0	0	0.29
2,4-Dimethyl-1-hexene	0	0	0	1.03
2-Amino-4,6-dimethoxypyrimidine	0.45	0	0	0
2-Butanone, 4-(4-hydroxy-3-methoxyphenyl)-	0	0	1.05	0
2-Octenal, (E)-	0	0	0	0.32
2-Propanol, 1,3-dichloro-	0	0	0	0.32
3',5'-Dimethoxyacetophenone	0	0	1.12	0

3,5-Heptanedione, 2,2,6,6-tetramethyl-	0	0.48	0	0
3-Amino-4-methoxybenzoic acid	0	0	0.85	0
3Beta-hydroxy-5-cholen-24-oic acid	0	0	0	1
4-Ethoxy-3-anisaldehyde	0	0	0.91	0
4-Ethylbenzoic acid	0	0	1.58	0
4-Hydroxy-3-methoxybenzyl alcohol	0	0	0.76	0
6,9,12-Octadecatrienoic acid, methyl ester	1.59	0	0	0
6-Tridecene, (Z)-	0	0	0	0.26
7-Hexadecenal, (Z)-	0	0	0	0.62
8,11-Octadecadienoic acid, methyl ester	5.02	0	0	0
9,10-Anthracenedione, 1,8-dihydroxy-3-methoxy-6-methyl-	0	0	2.32	0
9,10-Anthracenedione, 1,8-dihydroxy-3-methyl-	0	0	1.48	0
9,12-Octadecadienoic acid (Z,Z)-	0	0	0	3.54
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	2.81	1.12	0	0
9,12-Octadecadienoic acid, methyl ester, (E,E)-	4.76	0	0	0
9,12-Octadecadienoyl chloride, (Z,Z)-	0.27	0	0	0
9-Hexadecenoic acid, methyl ester, (Z)-	1.79	0	0	0
9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester	4.42	0	0	3.29

9-Octadecenoic acid (Z)-, methyl ester	20.7	8.99	0.73	0.94
9-Octadecenoic acid, (E)-	12.11	3.5	3.55	27.15
9-Tetradecenal, (Z)-	0	0.76	0	0
Benzaldehyde, (2,4-dinitrophenyl)hydrazone	0	0	1.84	0
Benzaldehyde, 4-hydroxy-3,5-dimethoxy-	0	0	2.4	0
Benzene, butyl-	0	0	0	1.06
Benzene, heptyl-	0	0	0	0.42
Benzene, hexyl-	0	0	0	0.77
Benzene, octyl-	0	0	0	0.23
Benzene, pentyl-	0	0	0	1.16
Benzene, propyl-	0	0	0	0.43
Benzoic acid, 4-methoxy-, methyl ester	0	0	1.24	0
Butorphanol	0	0	0.72	0
Cholesta-2,4-diene	0	0	1.11	0
Cyclohexanone, 4-(1,1-dimethylpropyl)-	0.31	0	0	0
Cyclopropaneoctanoic acid, 2-hexyl-, methyl ester	3.61	0	0	0
Cycloundecene, 1-methyl-	0.99	0	0	0
Decanedioic acid	0.34	0	0	0
Dibenz[d,f]cycloheptanone, 2,3,9-trimethoxy-	0	0	3.18	0
Dibutyl phthalate	0	1.46	0	0
Docosanedioic acid, dimethyl ester	0.39	0	0	1.17
Dodecanoic acid	0	0.95	7.51	0
Dodecanoic acid, methyl ester	0.19	0	0.8	0
Eicosane, 2-methyl-	0	0.46	0	0

Ethanamine, 2,2'-oxybis[N,N-dimethyl-	0	0	0	1.16
Ethanol, 2-(tetradecyloxy)-	0	0	0	0.65
Ethanone, 1-(3-methoxyphenyl)-	0	0	0.77	0
Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)-	0	0	5.74	0
Ethanone, 1-(4-hydroxy-3-methoxyphenyl)-	0	0	1.12	0
Ethanone, 1,1'-(1,4-phenylene)bis-	0	0.58	0	0
Ethanone, 1-[4-(1-methylethyl)phenyl]-	0	0	0	0.19
Ethyl Oleate	0	0	0	9.83
Fluridone	0	0	1.06	0
Furan, 2-pentyl-	0	0	0	0.41
Heneicosane	0	1.7	0	1.39
Heneicosanoic acid, methyl ester	0.2	0	0	0
Heptacosane	0	0.68	0.86	0
Heptadecane	0	0.88	0	0
Heptadecane, 3-methyl-	0	0.48	0	0
Heptadecanoic acid, methyl ester	1.02	0	0	0
Heptanoic acid, octyl ester	0.18	0	0	0
Hexadeca-2,6,10,14-tetraen-1-ol, 3,7,11,16-tetramethyl-, (E,E,E)-	0	0	0	0.21
Hexadecane	0	3.13	0	0
Hexadecane, 1-iodo-	0	0.65	0	0
Hexadecane, 2,6,10,14-tetramethyl-	0	0.77	0	0

Hexadecane, 2,6,10,14-tetramethyl-	0	0.85	0	0
Hexadecanoic acid, 14-methyl-, methyl ester	0.3	0	0	0
Hexadecanoic acid, 2-(octadecyloxy)ethyl ester	0	0	0	1.55
Hexadecanoic acid, ethyl ester	0	0	0	4.05
Hexadecanoic acid, methyl ester	11.23	3.76	2.86	0.55
Homovanillyl alcohol	0	0	2.77	0
Linoleic acid ethyl ester	3.79	0	0	0.36
Methyl tetradecanoate	1.51	0	0.7	0
Methylparaben	0	0	1.43	0
Nonadecane	0	1.96	0	0.59
Octacosane	0	2.13	0	1
Octadecanoic acid, 10-methyl-, methyl ester	0	0	0.95	0
Octadecanoic acid, ethyl ester	0	0	0	4.34
Octadecanoic acid, methyl ester	5.81	1.56	0	0
Octanal, 7-methoxy-3,7-dimethyl-	0	0	0	0.77
Oxacyclohexadecan-2-one	0	1.26	0	0
Oxiraneoctanoic acid, 3-octyl-, methyl ester, trans-	4.36	0	0	0
Pentacosane	0	0	2.3	0
Pentadecane	0	1.61	0	0
Pentadecanoic acid	3.67	15.21	13.36	23.79
Pentadecanoic acid, methyl ester	0.29	0	0	0
Phenol	0	0	1.59	0

Phenol, 2,6-dimethoxy-	0	0	5.01	0
Phenol, 2,6-dimethoxy-	0	0	0	0.17
Phenol, 2,6-dimethoxy-4-(2-propenyl)-	0	0	7.62	0
Phenol, 2-methoxy-4-(1-propenyl)-	0	0	0.99	0
Phenol, 2-methoxy-4-(1-propenyl)-, (E)-	0	0	0.92	0
Propofol	0	0.57	0	0
Quino[2,3-b]acridine-7,14-dione, 5,12-dihydro-	0	0	2.27	0
Squalene	0.34	9.17	2.81	0.34
Tetracosanoic acid, methyl ester	0.71	0	0	0
Tetradecane	0	1.53	0	0
Tetradecanoic acid	0	1.33	5.8	0.31
Tetratriacontane	0	5.8	0	2.34
Triacontanoic acid, methyl ester	1.97	0	0	0
Tricosanoic acid, methyl ester	0	0.54	0	0
Tridecanal	0	0	0	0.43
Vanillin	0	0	1.15	0