Australian Government

CSIRO

MEAT & LIVESTOCK AUSTRALIA

final report

Project code: B.CCH.7610

Prepared by: Chris McSweeney
Commonwealth Scientific and Industrial Research Organisation

Date published: 4 October 2016

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

Maximising energy-yielding rumen pathways in response to methane inhibition

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

Executive summary

Methane represents a direct loss of energy that could be used for increased production if the energy was redirected into live weight gain. Management of hydrogen and methane production in the rumen is an important factor to be considered, when developing strategies to reduce greenhouse gas emissions and improve efficiency of energy utilization from feed. This project has undertaken studies in cattle using a specific methanogen inhibitor (chloroform in a cyclodextrin matrix) to analyse the effect on methane production, metabolic hydrogen flux and subsequent responses in short chain fatty acid (SCFA) production, metabolites and rumen microbial community structure. The first *in vivo* experiment using three levels of chloroform-CD (low, medium and high dose) and two diets (hay and forage:concentrate diet), showed a progressively methane reduction when doses of chloroform were increased on both diets. Conversely hydrogen expelled by treated animals increased as methane production was reduced, with the greatest amounts of hydrogen loss occurring in animals supplemented with the hay/concentrate diet. The rumen fermentation parameters showed a shift in the fermentation pathways towards a more propionic acid production in the rumen of animals receiving chloroform with both diets. In relation to the rumen microbial community, there was a reduction in the relative abundance of some methanogen species (e.g *Methanobrevibacter*) when methane was reduced and an increase in *Prevotella* species. The microbial community PCoA plots show that samples from animals treated with chloroform were grouped separately from the control samples, showing a dose response. The results observed were in accordance with previous studies using bromochloromethane as antimethanogenic compound in small ruminants (Mitsumori et al., 2012). Therefore, dietary strategies were tested to facilitate the consumption of this hydrogen excess for improving energy supply to the animal.

The second *in vivo* trial to identify dietary supplements/microbial treatments that direct excess hydrogen in the rumen into energy yielding products was completed in 2014. Eight fistulated steers were fed a high fermentable concentrate diet with a ratio 60:40 hay:concentrate. The chloroform in a cyclodextrin matrix was provided progressively to the animals, after 21 days of treatment 4 animals received the supplement phloroglucinol + chloroform during 16 days, while the rest of the animals received the same dose of chloroform. After a 10 d wash out period (for phloroglucinol), 4 animals were inoculated in the rumen with an acetogenic bacteria + chloroform during five days, while the rest of the animals received the same dose of chloroform. Methane production (g) per kg of DMI was reduced and hydrogen expelled increased with the dose of chloroform as expected. Significant differences were observed on those animals treated with phloroglucinol + chloroform compared with the positive control group that received just chloroform. The hydrogen production (g) per kg of DMI was significantly decreased on the phloroglucinol group compared with chloroform group. The rumen fermentation parameters showed a shift towards more acetic acid production in the rumen of animals treated with phloroglucinol + chloroform. These results are in accordance with previous studies using phloroglucinol in ruminants, which observed an increased in acetic acid concentration (Tsai and Jones, 1975, Krumholz and Bryant, 1986, Murdiati et al., 1992). That increase suggested a redirection of the excess of hydrogen in the rumen towards acetic acid production by reductive dehydroxylation of phenolic compounds. In relation to the rumen microbial community, OTUs assigned to *Coprococcus* genus were positively increased by phloroglucinol treatment.

Coprococcus is likely to be an important player in the redirection of hydrogen towards acetic acid production by reductive dehydroxylation of phenolic compounds. Furthermore, an increase in daily weight gain in the animals treated with phloroglucinol was observed and might be an indication of hydrogen being redirected towards energy yielding pathways.

The third *in vivo* trial to identify dietary supplements/microbial treatments that direct excess hydrogen in the rumen into energy yielding products for the animal was completed in 2015. A slow fermentable forage (Rhode grass hay) was used as experimental diet to study the effect of phloroglucinol + chloroform on hydrogen flux, methane production, rumen fermentation and microbial population, following a similar experimental design as previous trial. Methane production (g) per kg of DMI was reduced as expected, however hydrogen expelled was not detected with the dose of chloroform used. Significant differences were observed on those animals treated with phloroglucinol + chloroform compared with the positive control group that received just chloroform. The rumen fermentation parameters showed a shift towards more acetic acid production in the rumen of animals treated with phloroglucinol + chloroform. An increase in daily weight gain was also observed in the animals treated with phloroglucinol confirming previous findings using the same compound with a different diet. The lack of effect on expelled hydrogen in chloroform groups could suggest a redirection of hydrogen which could be due to the high CP presented in the forage and the slower fermentable substrate.

The fourth and last in vivo trial to identify dietary supplements that direct excess hydrogen in the rumen into energy yielding products for the animal was completed. Twelve fistulated steers, fed with a low quality forage diet (Rhode grass hay), are being used to study the effect of different nitrogen sources (urea or casein) on hydrogen flux, methane production, rumen fermentation and microbial population when an antimethanogenic compound is applied (chloroform). After 21 days of treatment animals received a concentrate diet with a ratio 60:40 hay:concentrate to study the effects of supplements with a highly degradable diet. Animals expelled less hydrogen per mole of methane decreased when were fed with the hay diet compared with the faster fermentable diet. These results confirmed the hypothesis generated from previous trials about the amount of hydrogen expelled with high or low fermentable diets. Animals treated with casein and fed with hay showed no detrimental effects when methane was decreased by 90%. Urea showed promising results, regarding the hydrogen redirection, with the high fermentable diet. However, the experiment finalised with 3 animals per group because several animals stopped to eat due to the greater methane inhibition. Thus, the data analysis did not show significant results for some parameters although a pattern was observed with casein and urea for hay and hay:concentrate diet respectively.

In conclusion, the present project showed that a reduction in methane formation by 30-35% resulted in a redirection of H_2 into more reduced microbial end-products and eructation of excess hydrogen without an apparent adverse effect on DM intake, fibrolytic activity and general rumen function. The amount of expelled H_2 per mol of decreased methane was lower for the roughage hay diet suggesting a more efficient redirection of hydrogen, which due to the slower fermentation rate and evolution of H_2 compared with the hay:concentrate supplemented animals. The metabolomics analysis indicated that the nitrogen supply may have been enhanced by increased proteolysis and microbial protein synthesis particularly in

the methane inhibited cattle fed roughage hay. Although there was a redirection of H_2, dietary supplements or microbial treatments might be needed to drive the excess H_2 into energy-yielding substrates and consequently improve the energy supply to the animal. Supplementation of a phenolic compound in animals demonstrated a redirection of hydrogen excess towards acetic acid production when methanogenesis was inhibited, improving the energy supply to the animal. *Coprococcus* has been identified as one of the microorganism involved in that process. The significant increase in daily weight gain observed with some of the dietary treatments, which redirected hydrogen to energy yielding products, might improve the productivity by more than 10%. Future work should be focus on practical feeding farming strategies to capture this excess hydrogen as energy for the animal.

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1. Background

Reducing methane production could potentially improve productivity by nearly 5-10% for the same energetic intake by the animal provided rumen metabolism is not compromised. Several metabolic pathways within the rumen are involved in the conversion of feedstuffs into various energy-yielding products for use by the animal. Some of the hydrogen in the rumen is used in generating these energetic products but methanogens consume the greater majority of hydrogen to obtain energy for their metabolism and finally release methane. Management of hydrogen and methane production in the rumen is an important factor to be considered, when developing strategies to reduce greenhouse gas emissions and improve efficiency of energy utilization from feed.

This project will undertake studies in cattle using a specific methanogen inhibitor (chloroform in a cyclodextrin) to analyse the effect on methane production, metabolic hydrogen flux and subsequent responses in short chain fatty acid (SCFA) production and rumen microbial community structure.
It is hypothesized that:

(i) The rumen microbiota will adapt to the inhibition of methanogenesis and shift fermentation to reductive processes that will consume more reducing equivalents,
(ii) Excessive hydrogen gas will accumulate and not be captured as energy for the animal,
(iii) The consumption of excess hydrogen into energy-yielding substrates for the animal will require the provision of dietary supplements to drive hydrogen uptake or augmentation of minor hydrogenotrophic pathways such as autotrophic reductive acetogenesis.

This project is similar to the work conducted on bromochloromethane (BCM) inhibition of methanogenesis that was used in the Reducing Emissions from Livestock Research Program project published by Mitsumori et al*.,* 2012. Collaboration between CSIRO and the National Institute for Livestock and Grassland Science, Japan (NILGS) will be maintained to maximise project outputs. The project will complement the latest knowledge on nitrate supplementation by linking with the existing round 1 FtRG project “Practical and sustainable considerations for the mitigation of methane emissions in the northern Australian beef herd using nitrate supplements”. Furthermore intra-ruminal hydrogen and methane sensors which are currently being developed in the round 1 FtRG project “Measuring methane in the rumen under different production systems as a predictor of methane emissions” may be deployed in this project.

This project will be one of five projects that make up The Rumen Pan genome Program (RPP). The RPP will deliver new and emerging knowledge and strategies for reducing methane emissions from livestock while maintaining productivity and profitability. It will provide high quality data that will be used to deliver a comprehensive understanding of animal genotype x rumen environment x management interactions that drive methane emissions from livestock and potential strategies to reduce emissions. The project will also maintain collaboration between CSIRO and NILGS (RELRP: Microbial ecology of hydrogenotrophic rumen microorganisms in response to methane inhibitors) which identified that strategies need to be devised for the use of hydrogen in the methane inhibited rumen which will

require the provision of dietary supplements or stimulation of the hydrogen consuming pathways in bacteria.

2. Methods

Experimental design and sampling

Experiment 1. Eight fistulated Brahman steers (*Bos indicus*) were used in the first *in vivo* experiment. Animals were randomly allocated in two groups (4 animals per group), receiving a different ad libitum diet twice per day. One group was fed a forage only diet and the second group received a concentrate diet with a ratio 60:40 forage:concentrate (CSIRO's concentrate diet produced by Ridley AgriProducts Pty Ltd, Brisbane, Qld, Australia) (**Table 1**)

Table 1. Chemical composition of Rhode grass hay and concentrate (g kg^{-1} dry matter) and ingredients (g kg^{-1}) of concentrate.

Item	Hay	Concentrate
DM (g kg-1 fresh matter)	881	906
OM	802	841
NDF	765	263
ADF	454	120
ADL	64	30
CP	50	116
Ingredients		
Barley		574
Sorghum		200
Molasses mixer		30
Cotton hull pellet		100
Urea		5

Animals were adapted to each diet over an initial 17 d period. After that initial period, experimental animals were maintained in individual pens in an animal house for the measurement of individual intakes (10 d) and were treated with cyclodextrin (3 g/100 kg LW). On days 9 and 10 animals were confined in open-circuit respiration chambers for measurement of CH_4 and H_2 production and collection of rumen samples (control period). Following the initial adaption/control period animals received a low dose of chloroform-cyclodextrin (1 g/100 kg LW) for 10 d with the last two days being confined in open-circuit respiration chambers for direct measurement of CH_4 and H_2 production. Doses were then

increased to a mid level (1.6 g/100 kg LW) for 10 d with rumen fluid collection and CH_4/H_2 measurements, and then to a high level (2.6 g / 100 kg LW) for 10 d with a similar sampling regime for the final two days. The chloroform doses were split up in two shots and administered through the rumen cannula at 0 and 3 h after feeding. After a 15 d period without chloroform animals were returned to open circuit respiration chambers during two consecutive days with a similar rumen sampling regime (post-treatment period). The sequencing of events is shown in **Figure 1**.

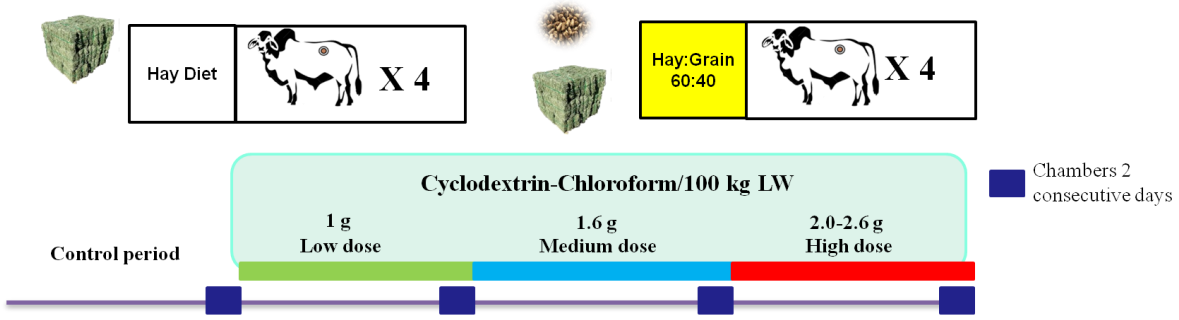


Figure 1. Dosing regimen for chloroform-CD complex.

Rumen fluid samples (60 mL per animal) were collected using a probe covered with two layers of cheesecloth at 3 h post feeding and just before dosing with chloroform during each confinement period in respiration chambers. Rumen samples were stored at −20◦C for short chain fatty acids (SCFA) and NH_3-N analyses. Additionally, 20 mL were kept at −80◦C for DNA extraction and metabolite analyses.

Experiment 2. Eight fistulated Brahman steers (*Bos indicus*) were used in the second *in vivo* experiment. Animals were randomly allocated in two groups (4 animals per group), receiving a concentrate diet with a ratio 60:40 forage:concentrate (CSIRO's concentrate diet produced by Ridley AgriProducts Pty Ltd, Brisbane, Qld, Australia).(**Table 2**)

Table 2. Chemical composition of Rhode grass hay and concentrate (g kg^{-1} dry matter) and ingredients (g kg^{-1}) of concentrate.

Item	Hay	Concentrate
DM (g kg-1 fresh matter)	917	887
OM	814	n.d
NDF	651	199
ADF	351	n.d.
CP	109	114
Ingredients		
Barley		458
Sorghum		200
Molasses mixer		30
Wheat		200

The treatments used were chloroform-cyclodextrin (6% w/w chloroform), phloroglucinol and an acetogenic bacteria. The phloroglucinol is considered a synthetic hydrogen sink and has been tested in ruminants (Tsai and Jones, 1975, Patel et al., 1981, Krumholz and Bryant, 1986, Murdiati et al., 1992). The acetogenic bacteria used for inoculate the animals was isolated from wallaby forestomach and showed excellent mixotrophic growth in the presence of high levels of hydrogen gas (Gagen et al., 2014).

Animals were adapted to the diet over a month. After that initial period, experimental animals were placed into individual pens in an animal house for the measurement of individual intakes (10 d) and were treated with cyclodextrin (2 g/100 kg LW). On days 9 and 10 animals were placed into open-circuit respiration chambers for measurement of methane and hydrogen production and collection of rumen samples. Following the initial adaption/control period animals received the chloroform-CD, increasing the dose progressively during 21 days to a mid dose level (1.6 g/100 kg LW, dose is based in previous trial) with the last two days confined in open-circuit respiration chambers for direct measurement of methane and hydrogen production and rumen fluid collection. After that period, one group received the chloroform-CD + Phloroglucinol for 16 days, whilst the other group just received the chloroform-CD. The final phloroglucinol dose was 75 g/100 kg LW (equivalent to 0.040 M rumen concentration). The last two days of that period rumen samples and methane and hydrogen measurements were taken as previously described. During the following 10 days both groups were treated with the same dose of chloroform-CD as wash out before received the second treatment, with a similar sampling regime for the final two days. After that period, 4 randomly animals were inoculated during 5 days with the acetogenic bacteria (700 mL of media per d) + chloroform-CD, whilst the other 4 animals were considered as the control group receiving the culture media without the microbial

treatment (700 mL per d) + chloroform-CD . During the last two days of the treatment animals were placed two consecutive days on chambers with a similar sampling regime as previously described. Finally, after a 14 d period receiving only chloroform-CD animals were placed in chambers during two consecutive days to study whether the acetogenic bacteria was established in the rumen of those animals previously inoculated. The sequencing of events is shown in **Figure 2**.

Rumen fluid samples (approx 60 mL per animal) were collected using a probe with 2 layers of cheesecloth through the cannula of the animal at 2 different sampling times (pre dose and at 3 h post dose) during confinement in respiration chambers to determine the effect on rumen fermentation parameters and rumen microbial communities. Rumen samples were stored at −20◦C for SCFA and NH_3-N analyses. Additionally, 20 mL were kept at −80◦C prior DNA and RNA extractions to determine stability in microbial communities and immediate effect on rumen function relative to each treatment event.

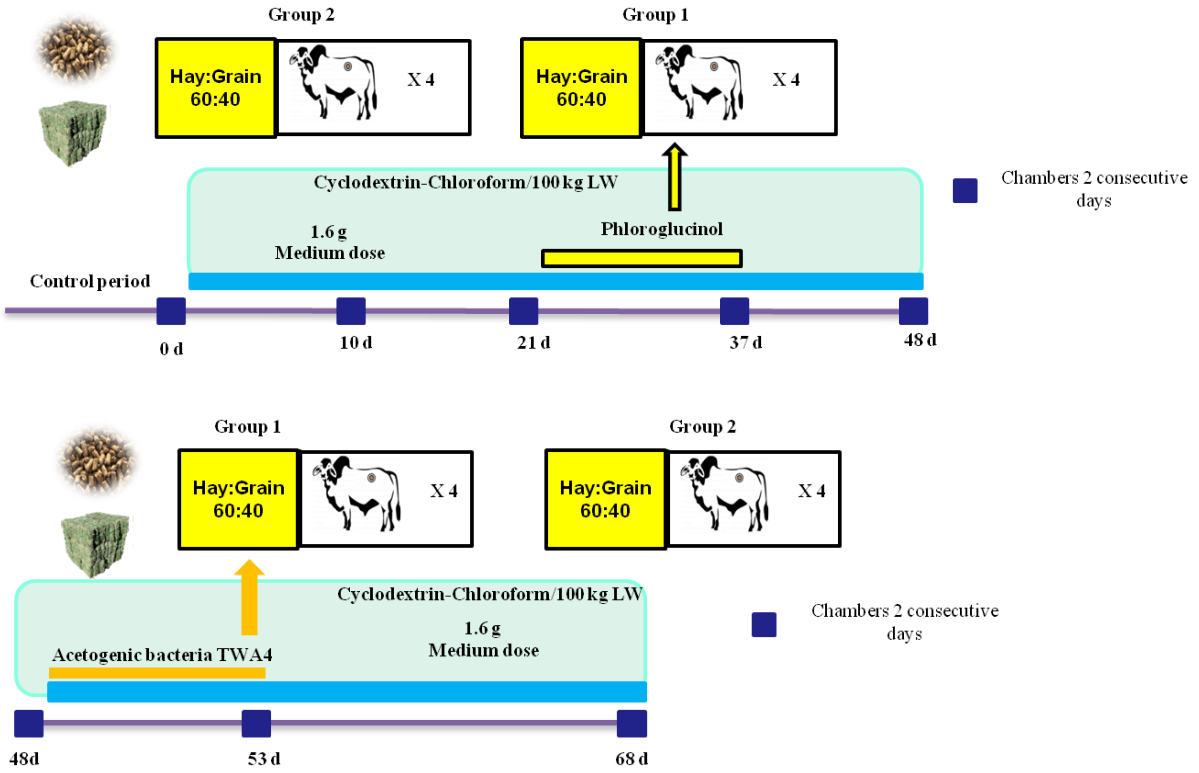


Figure 2. Dosing regimen for chloroform-CD complex and feed additives.

Experiment 3. Eight fistulated Brahman steers (Bos indicus) were used in the third *in vivo* experiment. Animals were randomly allocated in two groups (4 animals per group), receiving a forage diet (Rhode grass hay, Table 3).

Table 3. Chemical composition of Rhode grass hay.

Item	Hay
DM (g kg-1 fresh matter)	917
OM	806
NDF	661
ADF	359
ADL	46
CP	169

The treatments used were Chloroform-cyclodextrin (8% w/w chloroform) and phloroglucinol. The phloroglucinol is considered a synthetic hydrogen sink and has been tested in ruminants (Tsai and Jones, 1975, Patel et al., 1981, Krumholz and Bryant, 1986, Murdiati et al., 1992). Phloroglucinol was previously tested (trial 2) under a different diet showing promising results.

Animals were adapted to the diet over a month. After that initial period, experimental animals were placed into individual pens in an animal house for the measurement of individual intakes (10 d) and were treated with cyclodextrin (2 g/100 kg LW). On days 9 and 10 animals were placed into open-circuit respiration chambers for measurement of methane and hydrogen production and collection of rumen samples. Following the initial adaption/control period animals received the chloroform-CD, increasing the dose progressively during 21 days to a mid dose level (1.6 g/100 kg LW, dose is based in previous trial) with the last two days confined in open-circuit respiration chambers for direct measurement of methane and hydrogen production and rumen fluid collection. After that period, one group received the chloroform-CD + Phloroglucinol for 21 days, whilst the other group just received the chloroform-CD. The final Phloroglucinol dose was 90 g/100 kg LW (equivalent to 0.050 M rumen concentration). The last two days of that period rumen samples, methane and hydrogen measurements were taken as previously described. During the following 14 days both groups were treated with the same dose of chloroform-CD. Finally, after 14 days without treatment animals were placed in chambers during two consecutive days with similar sampling regime (During this period animals received cyclodextrin). The sequencing of events is shown in **Figure 3**.

Rumen fluid samples (approx 60 mL per animal) were collected using a probe with 2 layers of cheesecloth through the cannula of the animal at 2 different sampling times (pre dose and at 3 h post dose) during confinement in respiration chambers to determine the effect on rumen fermentation parameters and rumen microbial communities. Rumen samples were stored at −20◦C for SCFA and NH_3-N analyses. Additionally, 20 mL were kept at −80◦C prior DNA and RNA extractions to determine stability in microbial communities and immediate effect on rumen function relative to each treatment event. In addition, when animals were

placed in chambers, samples of Rhode grass hay were placed into nylon bags and incubated in the rumen of each animal for 24 and 48 h, to determine the ruminal degradation of DM.

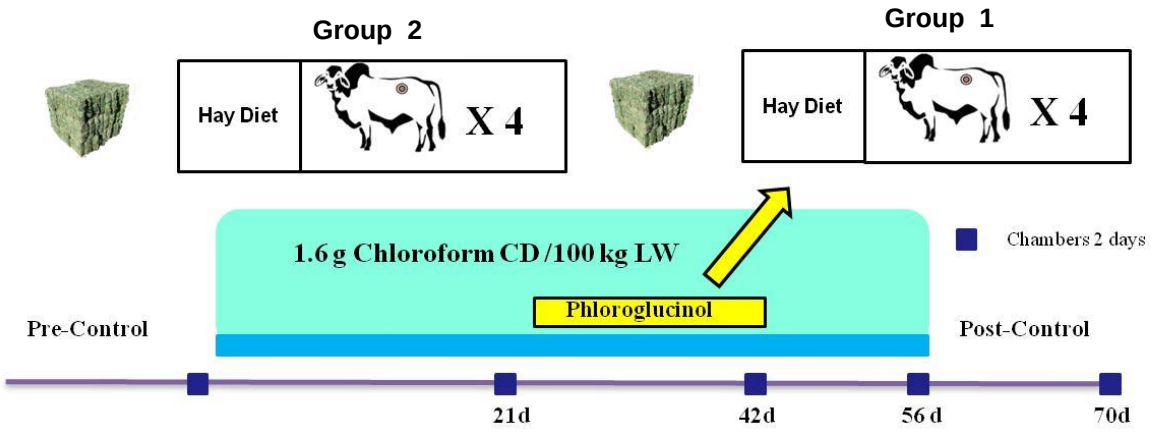


Figure 3. Dosing regimen for chloroform-CD complex and feed additive.

Experiment 4. Twelve fistulated Brahman steers (Bos indicus) were used in the fourth *in vivo* experiment. Animals were randomly allocated in three groups (4 animals per group), receiving a forage diet (first part of the trial) and a forage:concentrate diet (second part of the tiral) (Rhode grass hay, Table 4).

Table 4. Chemical composition of Rhode grass hay and concentrate (g kg^{-1} dry matter) and ingredients (g kg^{-1}) of concentrate.

Item	Hay	Concentrate
DM (g kg-1 fresh matter)	927	901
OM	946	945
NDF	785	218
Lignin	68	17
ADF	481	76
CP	80	124
Ingredients		
Barley		460
Sorghum		200
Soybean		80
Wheat		200

Animals were fed with a low quality forage diet (Rhode grass hay), and each group received one of the treatments: control, urea or casein to study the effects on hydrogen flux, methane production, rumen fermentation and microbial population when an antimethanogenic compound is applied (Chloroform-cyclodextrin (CD) (8% w/w chloroform)). After 21 days of treatment animals received a concentrate diet with a ratio 60:40 roughage:concentrate plus the treatment to study the diets effect.

Animals were adapted to the diet over a month. After that initial period, experimental animals were placed into individual pens in an animal house for the measurement of individual intakes (14 d) and were treated with cyclodextrin (2 g/100 kg LW). Since then, one group commenced with the urea treatment, the second group received the casein and the third group did not receive any treatment (control). The final urea dose was 130 g / animal/day (diluted in water and spray on the feed) and for the casein 300 g/animal/ day through the cannula. The doses were calculated to give to the animal a similar amount of N with both additives (50 g of N). Sulphur was supplemented to get the recommended S/N ratio of 0.07 for cattle. After 14 days animals were placed into open-circuit respiration chambers for measurement of methane and hydrogen production and collection of rumen samples. Following the initial adaption/control period the three groups received the chloroform-CD, increasing the dose progressively during 21 days to a mid dose level (1.6 g/100 kg LW, dose is based in previous trial) plus the feed additive (previously described) with the last two days confined in open-circuit respiration chambers for direct measurement of methane and hydrogen production and rumen fluid collection. After that period, animals stopped the chloroform treatment and were adapted to the concentrate receiving the urea and casein as previously described. Finally, after 14 days without treatment animals were placed in chambers during two consecutive days with similar sampling regime. Following the initial adaption/control period the three groups received the chloroform-CD, increasing the dose progressively during 21 days to a mid dose level (1.6 g/100 kg LW, dose is based in previous trial) plus the feed additive (previously described) with the last two days confined in open-circuit respiration chambers for direct measurement of methane and hydrogen production and rumen fluid collection as previously described. The sequencing of events is shown in Figure 4.

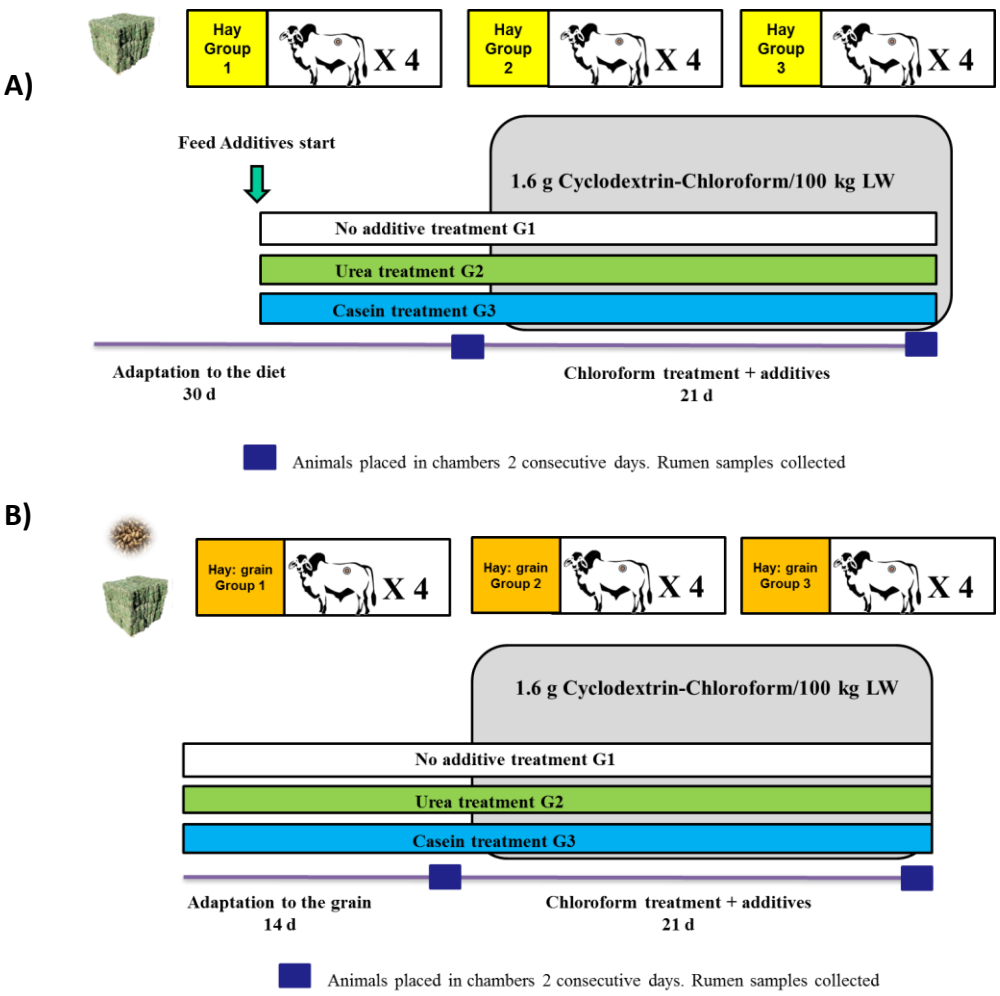


Figure 4. Dosing regimen for chloroform-CD complex and feed additives with hay (A) and hay:concentrate (B)diet.

Rumen fluid samples (approx 60 mL per animal) were collected using a probe with 2 layers of cheesecloth through the cannula of the animal at 2 different sampling times (pre dose and at 3 h post dose) during confinement in respiration chambers to determine the effect on rumen fermentation parameters and rumen microbial communities. Rumen samples were stored at −20◦C for SCFA and NH3-N analyses. Additionally, 20 mL were kept at −80◦C prior DNA and RNA extractions to determine stability in microbial communities and immediate effect on rumen function relative to each treatment event. In addition, when animals were placed in chambers, samples of Rhode grass hay or concetrate were placed into nylon bags and incubated in the rumen of each animal for 24 and 48 h, to determine the ruminal degradation of DM.

Gas measurements

Four open circuit respiration chambers were used to determine CH_4 production from individual steers. Each chamber had an internal volume of 23.04 m3 and was equipped with a water trough and feed bin containing the daily ration. Each chamber was maintained at 2.0 °C below ambient air temperature, approximately -10 Pa, and the relative humidity for the two, 24 h measurement periods varied from 50 to 75%. Air was drawn through a 250

mm diameter duct into each chamber at a rate of 3000 L/min. Exact flow rates, corrected to standard conditions for temperature and pressure (STP) for each chamber were used in calculations for CH_4 and H_2 production (Takahashi et al., 1999; Williams et al., 2007). Flow rate through each chamber was measured using thermal flow sensors (SS20.500 SCHMIDT® Flow Sensor). The air sample for the analysis of gas composition was drawn from a point in the exhaust duct through polyurethane tubing at 4.5 L/min using a micro diaphragm pump located between a multiport gas switching unit (SW & WS Burrage, Ashford Kent UK) and membrane drier (Perma Pure LLC). Air samples from each chamber initially passed through particulate filters (AF30-02 SMC Pneumatics Aust. Pty Ltd) and a four port fridge drier prior to the multiport gas switching unit which was programmed to cycle through each chamber and two outside air ports. Air samples passed through a chemical drier and were metered through independent rotameters before compositional analysis for CO_2 and CH_4 (Servomex 4100 Servomex Group Ltd. Crowborough, UK) and H_2 (Servomex Chroma, Servomex Group Ltd, Crowborough, UK; and Dräger X-am 5000, Draeger Safety Pacific Pty. Ltd., Victoria, Australia). Data for flow rate, temperature and chamber pressure, and CH_4 / H_2 content of the exhaust air for the final 315 s of each sampling event was used to calculate CH_4 and H_2 flux.

Chemical analysis

Feed samples were ground through a 1-mm sieve before analysis; DM, ash, NDF, ADF, lignin and total Nitrogen contents by Symbio Alliance (Eight Mile Plains, Queensland, Australia) following the accredited methods CF006.1, CF007, CF038.3, CF038.6 and CF003.2, respectively. The N values were converted to CP by multiplying by 6.25.

Concentrations of short chain fatty acids (SCFAs; acetate, propionate, n-butyrate, iso-butyrate, iso-valarete and n-valerate) were measured by gas chromatography (GC) as described by Gagen et al. (2014).

The NH_3-N concentration was determined by a colorimetric method following Chaney and Marbach (1962) method.

An UltiMate® 3000 HPLC system (Dionex, Sunnyvale, CA) with a dedicated Photodiode Array Detector and an Autosampler was used to determine the presence of formic acid in samples supernatant as described by Gagen et al. (2014).

Calculation of H_2 balance

The estimated recovery of metabolic hydrogen in the form of reduced protons (H) was calculated based on CH_4 production (MP; mol/day) and H_2 production (HP, mol/day), and the amount of acetate (C2), propionate (C3), butyrate (C4), isovalerate (Ci5) and valerate (C5) in the rumen (mM) for calculation of H utilised (HUr), H utilised in SCFA (HUSr), formate (HUFr), CH_4 (HUMr) and H_2 (HUHr) in the rumen (Goel et al., 2009, Mitsumori et al., 2012) described in the following equations:

$$\text{H produced in the rumen (HPr; mM)} = 2\,x\,C2 + C3 + 4\,x\,C4 + 2\,x\,Ci5 + 2\,x\,C5$$
$$\text{HUSr (mM)} = 2\,x\,C3 + 2\,x\,C4 + C5$$
$$\text{HUr} = (2\,x\,C3 + 2\,x\,C4 + C5) + (4\,x\,CH4) + (2\,x\,H2)$$

Recovery rate of metabolic hydrogen (HUr / HPr), is estimated as 0.9 (Moss et al., 2000). Therefore, HUMr (mM) and HUHr (mM) were estimated as:

$$HUr = 0.9\,x\,HPr = HUSr + HUFr + HUMr + HUHr \quad (1)$$

Here, HUHr was estimated based on H utilised in MP (HUMP; mol/day) and HP (HUHP; mol/day):

$$HUHr = HUMr\,x\frac{HUHP}{HUMP}$$

Thus, the equation (1) was expressed as:

$$\begin{aligned}\text{HUr} = 0.9\,x\,HPr &= HUSr + HUFr + HUMr + HUHr \\ &= HUSr + HUFr + HUMr + HUMr\,x\frac{HUHP}{HUMP}\end{aligned}$$

Here, (HUSr + HUFr) was expressed as HUSFr.

$$HUr = HUSFr + \left(1 + \frac{HUHP}{HUMP}\right)x\,HUMr$$

So,

$$HUMr = \frac{(0.9\,x\,HPr - HUSFr)}{\left(1 + \frac{HUHP}{HUMP}\right)} \quad (2)$$

Methane production of controls in the rumen (MPrcont; mmol/l) was calculated by using the equation 2.

$$MPrcont = \frac{HUMr}{4} = \frac{\left[\frac{(0.9\,x\,HPr - HUSFr)}{\left(1 + \frac{HUHP}{HUMP}\right)}\right]}{4}$$

Then, methane production at the low, mid and high doses of chloroform (MPrchl; mmol/l) in the rumen was calculated by using methane reduction rates (MR; %), which were calculated from methane production in respiration chambers (MP; mol/day), as:

$$MPrchl = MPrcont\ x\ MR\ x\ 100$$

Then, HPr was converted into total HP (tHP; mol/day) using a coefficient (K), which was a ratio of MP in the rumen (MPrcont or MPrchl) and MP in respiration chambers, as:

$$\text{Total HPr (tHP; mol/day)} = \text{K x HPr}$$

$$(K) = \frac{MP}{MPrcont\ or\ MPrchl}$$

Distribution of H in CH_4, H_2, SCFA and formate (%) were calculated as:

H in CH_4 $(HUM) = \frac{4\ x\ MP}{tHP\ x\ 100}$

H in H_2 $(HUH) = \frac{2\ x\ HP}{tHP\ x\ 100}$

H in SCFA and formate $(HUSF) = \frac{[0.9\ x\ tHP - (4\ x\ MP + 2\ x\ HP)]}{tHP\ x\ 100}$

H in SCFA $(HUS) = HUSF\ x\ \left(\frac{HUSr}{HUSFr}\right)$

H in formate $(HUF) = HUSF\ x\ \left(\frac{HUFr}{HUSFr}\right)$

Ruminal degradability of Rhode grass hay and concentrate

Ruminal degradability was measured on 3-g of 2 mm ground hay or concentrate. Samples were placed in 5 cm × 10 cm nylon bags with a pore size of 50 µm. Four bags were incubated in the rumen of each steer immediately before the morning feeding. Two bags were withdrawn after 24 h and the other two after 48 h of incubation. At the end of the corresponding incubation times bags were washed with cold water and maintained at −20◦C before being washed in a washing machine using a short cold water program including two bags with feed that had not been incubated in the rumen to account for solubility. After washing, bags were placed in the oven at 60◦C for 48 h. Ruminal degradability was calculated as the loss of dry matter over the corresponding incubation time.

Enrichment cultures for acetogenic bacteria

Enrichment culture medium was modified from the AC11.1 medium of Greening and Leedle (1989) and contained per litre, 38 ml of Mineral solution 2, 38 ml of Mineral solution 3, 200 ml of clarified rumen fluid, 1 ml of Pfennigs trace element solution and 1 ml of resazurin indicator as outlined by McSweeney et al. (2005) as well as 1.7 ml acetic acid, 0.6 ml propionic acid, 0.4 ml n-butyric acid, 0.1 ml of each of n-valeric, isovaleric, isobutyric and 2-methylbutyric acid, 2 g of $Na_2HPO_4.2H_2O$, 6 g of $NaHCO_3$, 0.5 g of yeast extract and 0.25 g of

cysteine-HCl. The final pH was adjusted to between 6.4 and 6.8. All media were prepared using standard anaerobic techniques (Hungate, 1969), were dispensed in an anaerobic chamber with an atmosphere of 95% CO2 and 5% H2 (COY Laboratory Products Inc., Ann Arbor, MI) and were sterilised by autoclaving at 121°C, 100 kPa for 20 minutes. Modified AC11.1 medium was dispensed either as 10 ml aliquots in 27 ml Balch tubes (Bellco, Vineland, NJ) or as 700 ml aliquots in 1250 ml bottles, both sealed with butyl rubber stoppers (Bellco). Glycerol was added at a final concentration of 10 mM and after inoculation tubes and bottle were then pressurized with H_2 to 100 kPa and incubated horizontally at 39°C.

Rumen metabolomics analyses

Samples from rumen fluid were prepared and metabolites quantified by Metabolomics Australia, University of Melbourne.

Amines quantification: Sample volumes of 10 µL of rumen fluid supernatant were placed in 2mL Eppendorf tubes under cold conditions (4^{o}C). Methanol (100% MeOH, 250 µL) containing four internal standards (^{13}C-sorbitol (0.5 mg/mL), $^{13}C_5$-^{15}N-Valine (0.5 mg/mL), 2-aminoanthracene (0.25 mg/mL) and pentafluorobenzoic acid (0.25 mg/mL)) was added to the sample tubes and the samples were vortexed for 5 min until uniform. The samples were then incubated in a Thermomixer (Eppendorf brand, distributed by Quantum Scientific, Australia) at 70^{o}C with a mixing speed of 850 rpm for 15 min, followed by a 15 min of centrifugation at 4^{o}C at 13,000 rpm (13,800 x *g*) in an Eppendorf benchtop centrifuge. The MeOH supernatant was transferred into a new 2 mL Eppendorf tube and set aside. Water (250 µL, Milli-Q grade) was added to the remaining sample pellet in the initial tube and vortexed for 5 mins before being centrifuged at 13, 000 rpm at 4^{o}C for 15 min. The H_2O supernatant was transferred to the Eppendorf tube containing the MeOH supernatant and vortexed to mix. A 10 µL aliquot was transferred to a fresh Eppendorf tube in preparation for derivatisation with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Aqc) followed by LC-MS analysis as per Boughton *et al.* (2011). In summary, 70 µL borate buffer was added to the sample aliquot, vortexed to mix and then centrifuged at 13, 000 rpm at 4^{o}C for 1 minute. 20 µL Aqc reagent was added to the sample aliquot, vortexed immediately to mix and then centrifuged at 13, 000 rpm at 4^{o}C for 1 minute. The Aqc-treated samples were incubated in a Thermomixer at 55^{o}C with a mixing speed of 1150 rpm for 10 min and then centrifuged at 13, 000 rpm at 4^{o}C for 5 min. The derivatized samples were then transferred to HPLC vials for LC-MS analysis as described by Boughton *et al*.(2011).

Sugars, organics and fatty acids quantification: Sample volumes of 240 µL of rumen fluid supernatant were placed in 2mL Eppendorf tubes under cold conditions (4^{o}C) and 320 µL of 100 % methanol, containing 2% 13C6 Sorbitol as a quantitative internal standard, was added. The samples were then vortexed for 1 min and incubated with shaking (950 r.p.m.) at 30oC for 15 mins prior to centrifugation at 13,000 rpm for 15 min at room temperature in a bench-top Eppendorf centrifuge. The supernatant was transferred to a clean tube while the remaining pellet was re-extracted with 640 µL of $CHCl_3$ and vortexing for 1 min. After re-centrifugation as before, 60 µL of the polar upper phase was removed and dried under vacuum without heating prior to preparation for sugars and organic acids quantitation as described by Dias *et al*. (2015). The lower $CHCl_3$-phase was dried under vacuum without heating then reconstituted in 320 µL of $CHCl_3$ before a 160 µL aliquot transferred to a glass

insert and re-dried under vacuum prior to preparation for fatty acid quantitation described by Dias *et al. (2015)*.

DNA extractions

DNA extractions were carried out on rumen samples using the cetyltrimethylammonium bromide (CTAB) method of Brookman et al., (2005) with minor modifications as follows: samples were centrifuged (13,000 X g for 5 min), and the supernatant was removed before DNA extraction. Cells were homogenized with 200 mg of silica-zirconium beads (1:1 mixture of 0.1- and 1.0-mm beads; Biospec, Bartlesville, OK) and 800 µl of CTAB buffer in a Mini-Beadbeater-8 (Biospec) on maximum speed for 2 min, twice. Samples were incubated at 70°C for 20 min and centrifuged at 10,000 X g for 10 min, and the supernatant was mixed with 500 µl of 25:24:1 phenol-chloroform-isoamyl alcohol (Fluka BioChemika, Buchs, Switzerland).

Real-Time PCR Analysis

The DNA samples were used as templates for quantifying the abundance of three cellulolytic rumen bacterial species, *F. succinogenes*, *R. albus* and *R. flavefaciens*, anaerobic rumen fungi, protozoa populations and the formyltetrahydrofolate gene for acetogens. The primers and assay conditions used were previously published (Denman and McSweeney, 2005, Koike and Kobayashi, 2001, Denman and McSweeney, 2006, Sylvester et al., 2004). Real-time PCR analyses were performed per triplicate on an Applied Biosystems™ ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific Inc.). Assays were set up using the SensiFAST SYBR® Lo-ROX (Bioline). Optimisation of assay conditions was performed for primer, template DNA and $MgCl_2$ concentrations. An optimal primer concentration of 400 nM, a final $MgCl_2$ concentration of 3mM and DNA template concentration of 50 ng were used for each assay under the following cycle conditions: one cycle of 50^{o}C for 10 s and 95^{o}C for 2 min 30 s for initial denaturation, forty cycles at 95^{o}C for 15 s and 60^{o}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^{o}C/s from 60 to 95^{o}C. Estimation of abundance of target populations was calculated using standard curves for each target gene generated from cloned PCR products (Mitsumori et al., 2012).

16S rDNA Analysis

Using high throughput sequencing platforms and barcode, phylogenetic based methods targeting the 16S rDNA gene were used to deeply characterise the microbial populations present in the rumen for the control and treatment periods. The V4 region of the 16S rRNA gene was targeted using specific primers GTGCCAGCMGCCGCGGTAA-Forward and GGACTACHVGGGTWTCTAAT-Reverse. Each individual DNA sample was amplified using the specific primers and a unique barcode. After amplification products were visualised by performing gel electrophoresis. Product quantities were calculated and an equal molar amount of each product was pooled. The pooled products were run in a 1.5 % agarose gel and the product gel extracted and purified prior to submission for Illumina Miseq sequencing.

Short read sequence data generated was analysed using the QIIME: Quantitative Insights Into Microbial Ecology software package (Caporaso et al., 2010). Sequences were then de-multiplexed in QIIME based on their unique barcode, clustering of sequences to OTUs of 97%

similarity were performed using uclust (Edgar, 2010). Taxonomic assignment of sequences was performed against the Greengenes database (McDonald et al., 2012) using the RDP classifier software (Wang et al., 2007). Additional analysis of OTU's was performed in the R packages ade4, Phyloseq and DESeq2 (Chessel et al., 2004, McMurdie and Holmes, 2013, Love et al., 2014).

Statistical Analyses

Data in experiment 1 were analysed as a repeated-measures analysis using the GLM procedure of SPSS (IBM, version 21.0), with the animal as the experimental unit. The effect of dose was analysed for CH_4 / H_2 production, DMI, VFA, ammonia and pH. Metabolites were analysed as univariate model using the GLM procedure of SPSS. Data from experiment 2 and 3 were analysed as a univariate model using the GLM procedure of SPSS, 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 production, DMI, VFA, ammonia and pH. Effects were considered significant at $P \leq 0.05$. When significant differences were detected, differences among means were tested with the least significant difference (LSD) comparison test. An analysis of variance, done with R software, was used to establish differences in microbial concentration (qPCR) caused by the study doses/treatments.

3. Results

Experiment 1

Ruminal fermentation and gas production

Dry matter intakes (DMI, kg/d) on both diets were not significantly affected when animals were treated with increasing dosages of chloroform (Table 5). CH_4 production (g) per kg of DMI was reduced significantly ($P < 0.01$) for mid and high doses compared with the control on both diets. Conversely H_2 expelled by treated animals increased significantly ($P < 0.05$) as CH_4 production was reduced, with the greatest amounts of H_2 release (g/Kg DMI) occurring in animals supplemented with the roughage hay:concentrate diet. In addition the amount of H_2 expelled relative to the decrease in CH_4 (mol H_2/ mol CH_4 reduction) was greater in animals fed the concentrate diet. Fourteen days after the chloroform treatment was terminated, the CH_4 and H_2 production were similar to the control period for both diets (data not shown).

Rumen SCFA analysis showed a shift in the fermentation pathways (Table 5) toward a higher propionate profile when chloroform was increased on both diets. This was reflected by a significant decrease in acetate ($P < 0.001$) and an increase in propionate ($P < 0.05$) for both diets. As a result, a significant decrease of the acetate:propionate ratio was observed at mid and high doses for hay:concentrate and at all the doses for hay diet. Total SCFA concentration was similar between control and chloroform doses for the hay:concentrate diet. The hay diet however, showed a significant ($P < 0.001$) increase in total SCFA concentration when animals were treated with the low and medium dose of chloroform and a decrease with the high dose at 3 h post feeding. Butyrate increased significantly with chloroform in the roughage diet ($P < 0.01$), while the branched-chain SCFA increased with

both diets. Rumen pH post feeding was significantly increased with chloroform ($P < 0.001$) treatment for both diets. Rumen ammonia concentration increased significantly ($P < 0.05$) compared with the control period in animals fed the roughage hay diet and treated with chloroform. A significant increase in formate was observed for all chloroform doses for the concentrate diet and only with the hay diet at the highest chloroform dose.

Rumen metabolite profiles for control and treated animals at mid dose of chloroform is shown in table 6. Some amino acids, organic acids and sugars were significantly increased on both diets in animals treated with chloroform, although the profile observed was different for each diet. A greater number of metabolites increased significantly ($P < 0.05$) for those animals fed the hay diet, (amino acids: serine, homoserine, asparagine, threonine, proline, valine, ilsoleucine, leucine, tyramine and phenetylamine; the organic acids: malate, fumarate, malonate and nicotinic acid; and the sugars: arabitol, fructose and inositol). In animals fed the hay:concentrate diet the greatest increase ($P < 0.05$) was observed for the amino acids: homoserine, asparagine, proline, valine, isoleucine, glutamate ($P = 0.053$), and leucine ($P = 0.080$); and the sugars: ribose, arabitol and inositol. A marked fold increase was observed in nucleic acid precursors/derivatives (inosine and hypoxanthine) in chloroform treated animals compared with the control group, fed with hay ($P \leq 0.05$) or hay:concentrate ($P \leq 0.10$) diet, while no significant effect was observed on lactate concentrations (Table 7).

Table 5. Control and chloroform doses (low, mid and high) effects on DMI, CH_4 and H_2 production, and rumen fermentation parameters from samples collected 3 h after feeding of animals fed with hay:concentrate or hay diet.

	Hay:concentrate diet						Hay diet					
	Control	Low	Mid	High	SEM	P-value	Control	Low	Mid	High	SEM	P-value
DMI kg	5.9	5.8	5.6	5.0	0.11	0.178	4.2	4.6	4.4	4.7	0.21	0.139
CH_4 (g/kg DMI)	24^{a}	19^{ab}	15^{b}	10^{b}	0.79	0.002	27^{a}	21^{a}	17^{b}	12^{b}	2.27	0.004
H_2 (g/kg DMI)	0.0^{c}	0.97^{b}	1.4^{a}	3.2^{a}	0.11	0.022	0.0^{c}	0.33^{b}	0.83^{b}	1.7^{a}	0.14	0.015
mol H_2/ mol CH_4 reduced*	-	1.7	1.4	1.6	0.23	0.927	-	0.66	0.63	0.89	0.17	0.817
pH	6.4^{b}	6.6^{a}	6.6^{a}	6.7^{a}	0.01	0.001	6.6^{c}	6.7^{ab}	6.8^{a}	6.7^{b}	0.05	0.001
Formate (mM)	0.0^{c}	4.2^{b}	8.5^{a}	12^{a}	0.41	0.001	0.0^{b}	0.0^{b}	0.0^{b}	4.2^{a}	0.19	0.011
NH_3-N (mg/100 mL)	4.4	2.8	3.1	4.2	0.45	0.622	2.3^{a}	4.7^{b}	4.8^{b}	5.2^{b}	0.32	0.028
Total SCFA, (mM)	88	81	79	79	1.9	0.281	79^{b}	91^{a}	82^{ab}	66^{c}	3.80	0.001
Fatty acid (mol/100 mol)												
Acetate	62^{a}	56^{b}	55^{b}	53^{b}	0.50	0.001	67^{a}	62^{b}	61^{b}	58^{c}	1.04	0.001
Propionate	18^{b}	21^{b}	21^{b}	23^{a}	0.40	0.034	15^{b}	18^{a}	18^{a}	19^{a}	0.63	0.002
i-Butyrate	1.5^{c}	1.7^{ab}	1.8^{b}	1.9^{a}	0.03	0.002	1.7^{c}	1.7^{c}	1.9^{b}	2.1^{a}	0.05	0.001
Butyrate	12	13	14	13	0.33	0.112	9^{b}	11^{a}	11^{a}	12^{a}	0.32	0.002
i-Valerate	3.1^{c}	3.8^{b}	4.4^{b}	4.7^{a}	0.11	0.001	3.2^{c}	3.3^{c}	3.9^{b}	4.8^{a}	0.18	0.001
Valerate	3.4^{b}	4.0^{a}	4.0^{a}	4.1^{a}	0.07	0.012	3.6^{b}	3.8^{b}	4.0^{b}	4.7^{a}	0.16	0.001
A:P	3.4^{a}	2.6^{a}	2.5^{b}	2.3^{b}	0.07	0.004	4.4^{a}	3.5^{bc}	3.4^{b}	3.0^{c}	0.18	0.001

*Taken into account the DMI per animal

SEM: Standard error of the mean.

$^{a–c}$Within a row treatment means for each diet without a common superscript differ, $P < 0.05$.

Table 6. Chloroform (mid dose) effects on rumen metabolites (picomoles/mL rumen fluid) on animals fed at hay:concentrate or hay diet 3 h post feeding.

	Hay:Concentrate				Hay			
Metabolites	**Control**	**Mid dose**	**SEM**	***P*-value**	**Control**	**Mid dose**	**SEM**	***P*-value**
Amino Acids & Amines								
Asn	0.58	0.58	0.09	*n.s.*	0.22	0.43	0.05	*
Ser	11	14	1.4	*n.s.*	5.4	7.6	0.56	*
Homoserine	2.6	4.3	0.43	*	1.0	3.0	0.43	**
Gly	13	20	2.6	*n.s.*	9.2	13.4	1.2	**
Ala	0.32	0.27	0.13	*n.s.*	0.63	0.04	0.16	*t*
Asp	26	56	7.4	*	18	53	7.4	**
Glu	54	78	6.1	*t*	53	61	4.4	*n.s.*
Beta-Ala	3.1	4.3	0.71	*n.s.*	1.2	3.1	0.53	*t*
Thr	8.7	12	1.1	*n.s.*	4.4	7.6	0.67	**
GABA	3.0	3.3	0.42	*n.s.*	1.5	2.7	0.27	**
Pro	13	21	2.0	*	8.1	15.7	1.7	**
Lys	18	30	3.6	*n.s.*	9.8	15	1.3	*
Putrescine	15	11	1.8	*n.s.*	3.5	2.6	0.25	*t*
Tyr	2.8	4.6	0.65	*n.s.*	1.5	2.6	0.23	**
Met	3.9	5.5	0.52	*n.s.*	2.0	3.1	0.22	***
Val	8.9	24	3.8	*	4.2	11	1.4	**
Tyramine	1.9	1.8	0.17	*n.s.*	0.2	1.4	0.27	*
Ile	5.1	12	1.9	*	2.1	6.0	0.80	***
Leu	5.5	12	1.9	*t*	2.5	5.6	0.62	***
Phe	3.2	6.2	1.00	*n.s.*	1.2	2.5	0.25	***
Trp	0.97	1.1	0.05	*n.s.*	0.5	0.7	0.04	*

Phenethylamine	0.78	0.87	0.17	*n.s.*	0.6	3.7	0.62	***
Tryptamine	0.35	0.40	0.05	*n.s.*	0.02	0.29	0.06	**
Organic Acids								
Malonate	19.0	22.7	2.2	*n.s.*	19.9	26.4	1.4	**
Nicotinic Acid	9.1	11.3	1.2	*n.s.*	4.5	6.9	0.55	*
Fumarate_2TMS	1.9	2.4	0.33	*n.s.*	1.45	2.50	0.20	***
Pipecolate_2TMS	0.8	3.2	0.70	*t*	0.92	2.53	0.39	*
Malate	4.2	12.4	3.1	*n.s.*	3.8	6.2	0.50	**
Salicylate	2.6	2.5	0.28	*n.s.*	3.1	4.5	0.32	**
Sugars								
S1_Xylose	2.5	2.5	0.13	*n.s.*	2.0	2.5	0.10	***
Xylose_2	1.5	1.5	0.10	*n.s.*	1.2	1.7	0.10	***
S3_Ribose	3.5	4.8	0.28	**	4.8	5.7	0.20	*
Xylitol	0.5	0.6	0.03	*n.s.*	0.56	0.72	0.04	**
S2_arabitol	2.1	4.5	0.46	***	4.6	6.6	0.47	*
Fucose MX1	1.0	0.9	0.06	*n.s.*	0.78	0.88	0.03	*t*
Fructose 1	6.4	8.6	0.92	*n.s.*	1.45	2.28	0.21	*
S4_Galactose	0.6	0.7	0.05	*n.s.*	0.49	0.64	0.03	***
S3_Inositol	0.46	1.13	0.14	**	0.30	0.67	0.09	*
Uric acid	2.33	1.95	0.11	*t*	2.09	1.90	0.06	*n.s.*
Ferulic Acid	2.8	2.8	0.20	*n.s.*	3.15	3.57	0.12	*t*
S2_Glucose-6-P MX1	7.3	3.5	0.98	**	3.50	3.85	0.14	*n.s.*
S1_Sucrose	0.63	0.70	0.08	*n.s.*	0.76	1.07	0.09	*t*

*** ($P < 0.001$); ** ($P < 0.01$); * ($P < 0.05$); *t* ($P < 0.1$); *n.s.* ($P > 0.1$)

Table 7. Chloroform (mid dose) effects on metabolites profile (x-fold) on animals fed at roughage or Hay:concentrate diet 3 h after feeding.

	Hay:Concentrate			Hay		
	Control	Mid dose	SEM	Control	Mid dose	SEM
Organic Acids						
Lactic acid	1	0.27	0.31	1	1.2	0.10
Fatty Acids and sterols						
Hexanoic acid	1	2.6^{t}	0.21	1	2.5*	0.19
Heptanoic acid	1	5.0^{t}	0.30	1	4.1*	0.27
Heptadecanoate	1	1.2	0.15	1	1.7*	0.12
Octadecanol	1	2.2**	0.10	1	2.6***	0.04
Nucleic acids				1		
Inosine	1	4.4^{t}	0.30	1	2.2*	0.17
Hypoxanthine	1	21^{t}	0.48	1	5.0***	0.25

*** (P < 0.001); ** (P < 0.01); * (P < 0.05); t (P < 0.1)

Polynomial and linear regression analyses for roughage and concentrate diets respectively, revealed a strong correlation between the moles of CH_4 produced and the percentage of metabolic H_2 recovered in SCFA, CH4, formate or expelled H_2 (Figure 5) for both diets. However each diet showed a different pattern for the predicted H_2 in SCFA; the highest percentage of metabolic H_2 recovered from SCFA for the hay:concentrate treatment occurred at the high dose of chloroform, for the hay diet it was predicted at the mid dose, but declined for the high dose.

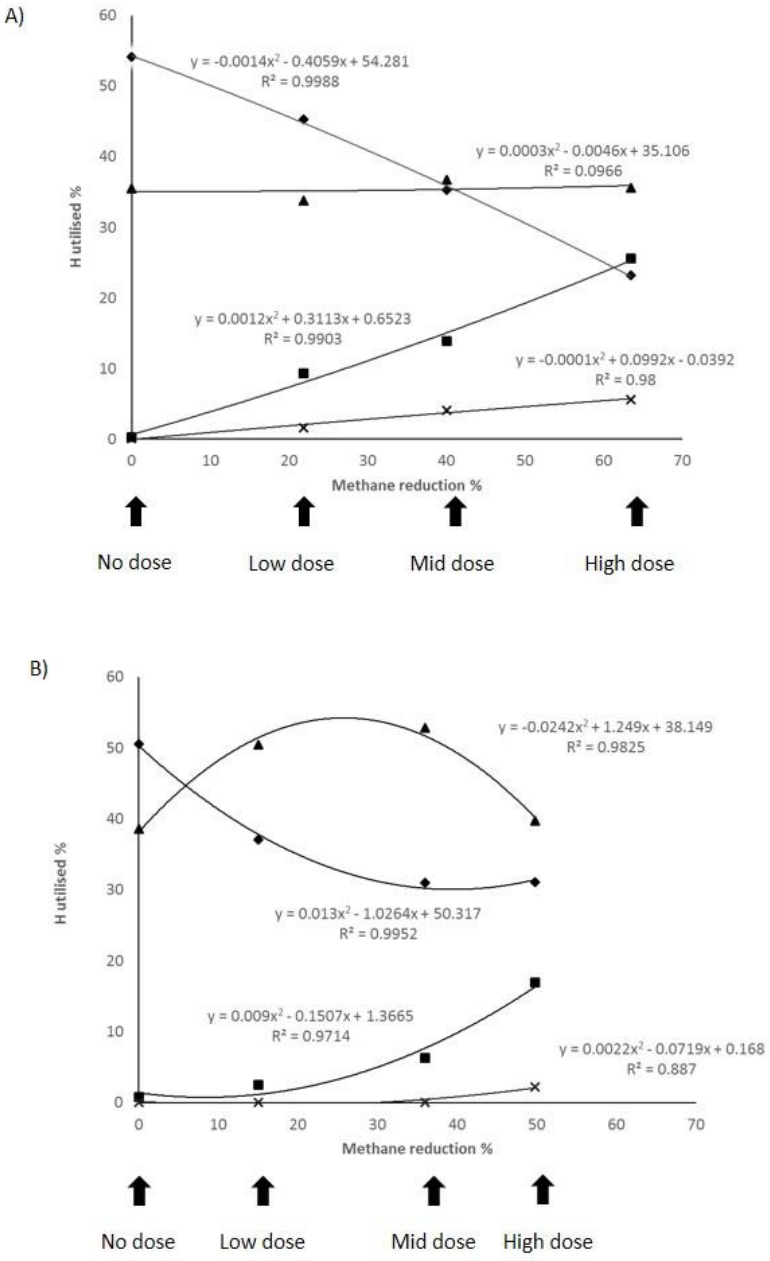


Figure 6. Distribution of metabolic H_2 utilisation (H) in SCFA (▲), CH4 (♦) H2 (■) or formate (X) at various levels of chloroform (zero, low, mid and high; from left to right) in animals fed with hay:concentrate diet A) or hay diet B).

Microbial community

The dose effect of chloroform on the abundance of methanogens, protozoa and fungi are shown in figure 6 for hay:concentrate and hay diet respectively. The methanogen abundance decreased ($P < 0.05$) with increasing doses of chloroform for both diets. The chloroform doses tended to increse the protozoa abundance in animals fed with the hay:concentrate diet, whereas the anaerobic fungi were not significantly affected by the chloroform doses for either diets.

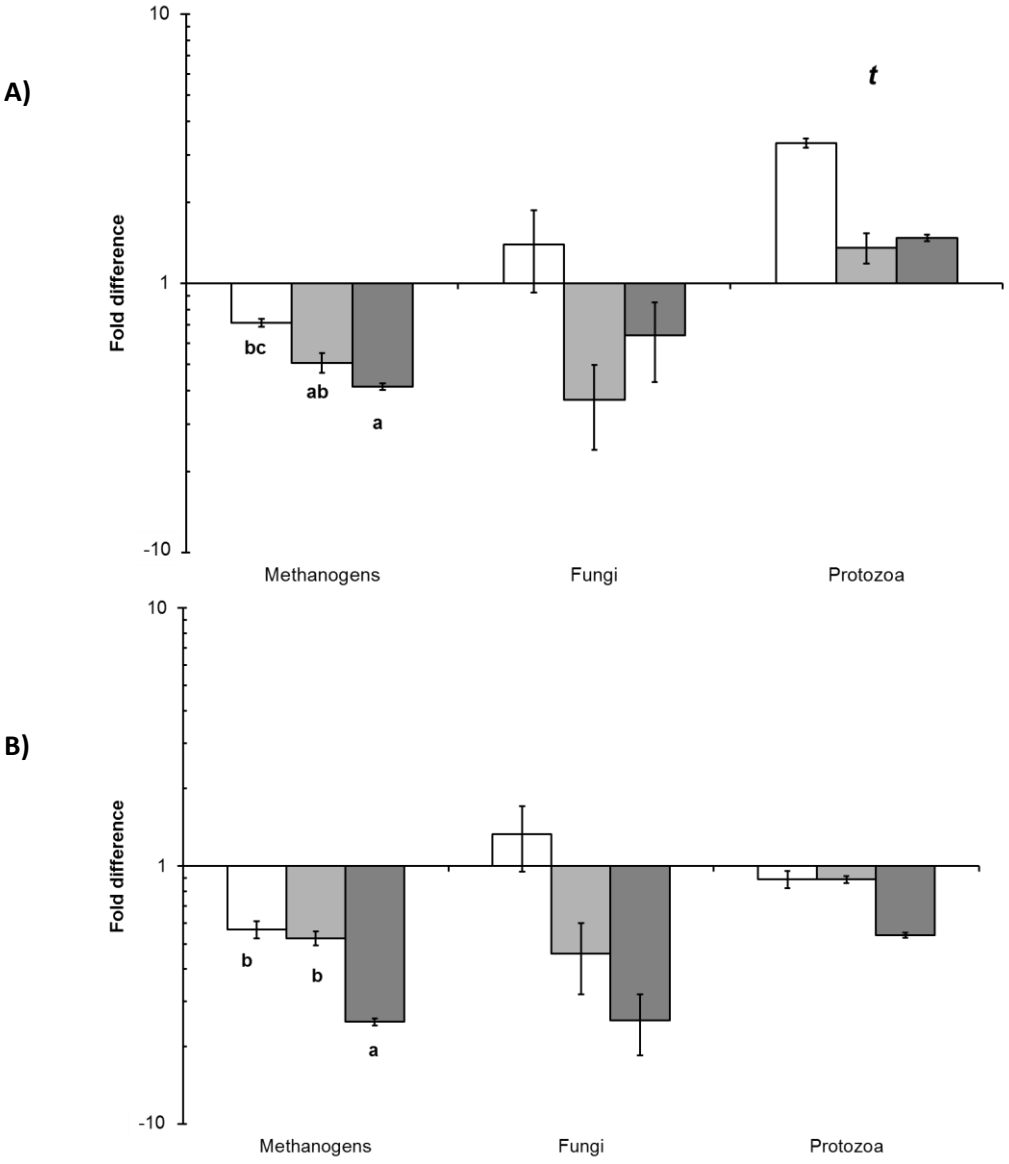
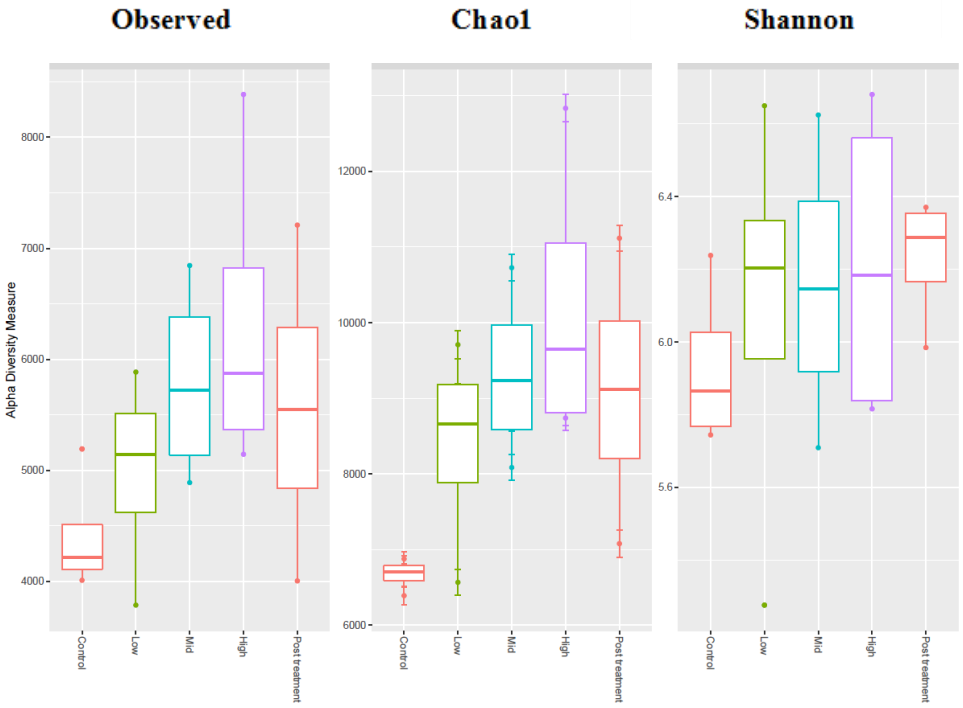


Figure 6. Quantitative PCR analysis of methanogens, protozoa and anaerobic fungi population changes in response to doses of chloroform (low ; white, mid; grey and high; black) in animals fed a hay:concentrate diet a) or hay diet b). [a,b] Letters denote significant differences between treatments, bars that do not share the same letter for a species are significantly different from each other ($P < 0.05$). [t] Denote a trend between treatments ($P < 0.1$). The y-axis denotes fold change from control period.

The diversity analysis of the rumen microbiota showed that total microbial species richness was impacted with the administration of all chloroform doses in animals fed the hay:concentrate diet, whilst only the highest dose of chloroform had a significantly altered for the hay diet. While the chloroform caused an increase in observed and estimated species richness for concentrate diet animals, a contraction in shannon diversity was observed for mid and high doses on a roughage diet. The microbial species richness from the post-treatment period samples showed similar values to the highest dose of chloroform on both diets (Figure 7).

A)



B)

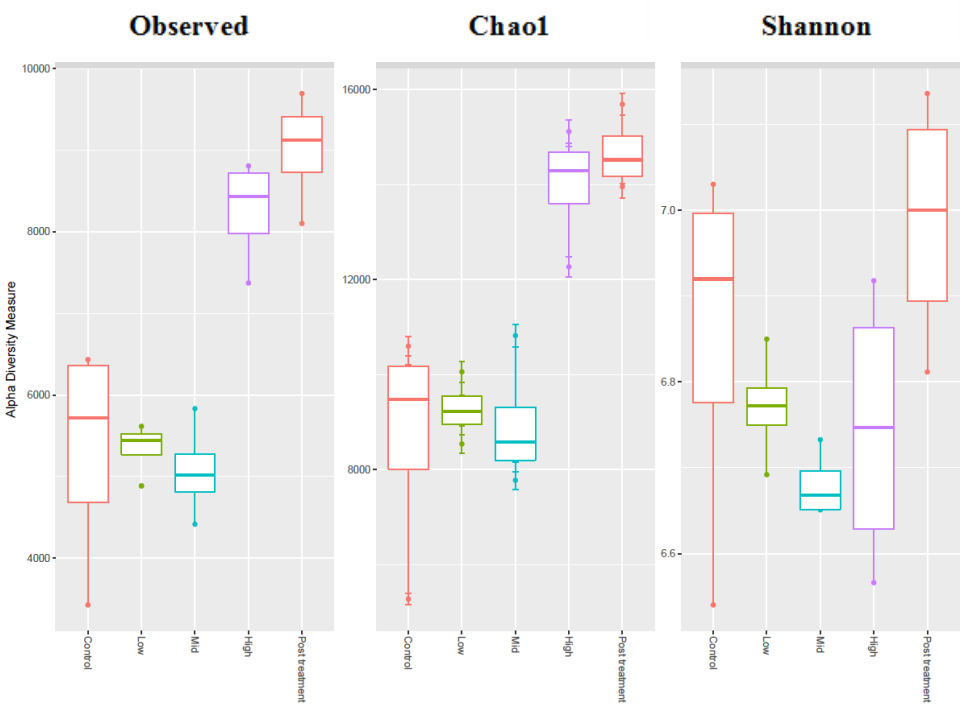


Figure 7. Alpha diversity measures for rumen microbiomes fed with hay:concentrate (A) or hay (B) diets at control, post-treatment, low, mid, and high doses of chloroform illustrating the total observed taxonomic units (Observed), the Chao1estimates (Chao1) and the Shannon diversity index (Shannon). Boxplots indicate variance within the sampled animals with the box boundaries showing the first and third quartiles, the median value indicated as a horizontal line and the whiskers extend to 1.5 times the interquartile range.

The structure of the microbiomes as determined by beta diversity analysis showed a clear separation between the control and chloroform doses for both diets (Figure 8). The post-treatment period was similar to the low chloroform dose or intermediate between that dose and the control treatment. The greatest variance observed between control and chloroform groups was 18 % and 15% in concentrate and roughage diets respectively. Variation between animals explained the next level of variance irrespective of treatment due to one animal possessing a different microbial population compared to the other three animals on both diets. The variance between individual animals decreased at the high chloroform dose and post-treatment period.

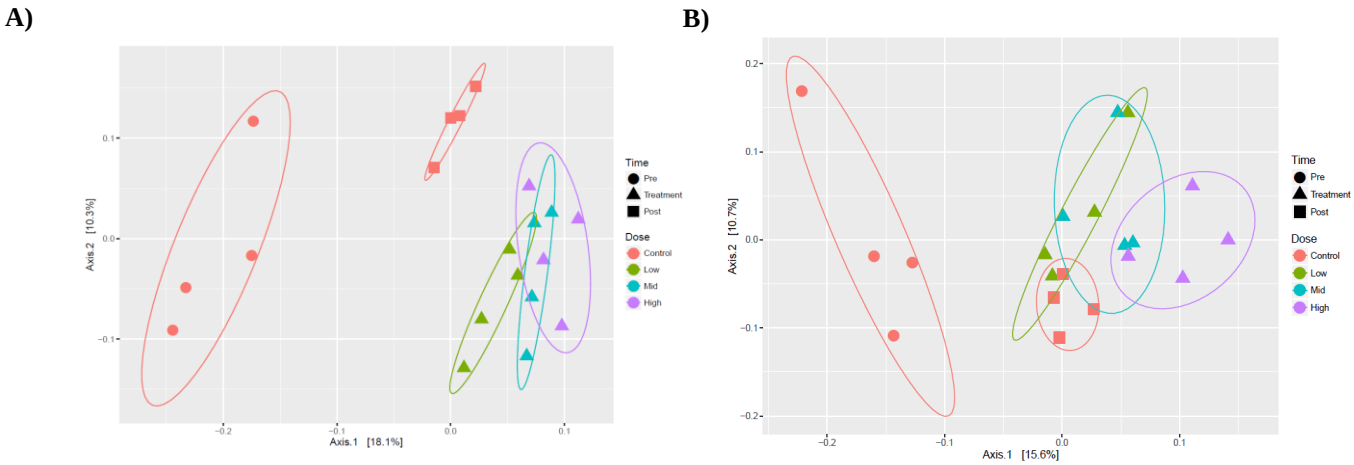


Figure 8. Principle Coordinate Analysis comparing changes in microbial OTU classification based on unweighted Unifrac calculations for control (●), post-treatment (■) and chloroform (▲) treatments at low (Greening and Leedle), medium (blue) and high (purpure) doses of chloroform in animals fed with hay:concentrate (A) or hay (B) diets.

Analysis of the rumen microbiome showed a shift in the relative abundance at the phylum level when the chloroform dose was increased in both diets. An increase in the sequences assigned to the Bacteroidetes phylum and a decrease in Firmicutes, Synergistetes, Verrucomicrobia and archaea were observed in both diets (Figure 9 and 10). An increase in sequences assigned to the Proteobacteria phylum, mainly classified as Succinivibrionaceae family, were observed in the hay:concentrate diet animals upon treatment with chloroform in contrast with hay diet. Consequently the Bacteroidetes:Firmicutes ratio increased with both diets when methanogenesis was inhibited (Figure 11). The ratios of sequences assigned to archaea, Synergistetes and Verrucomicrobia in relation to bacteria, decreased when chloroform dose increased and methane was inhibited showing a dose-dependent effect (Figure 12).

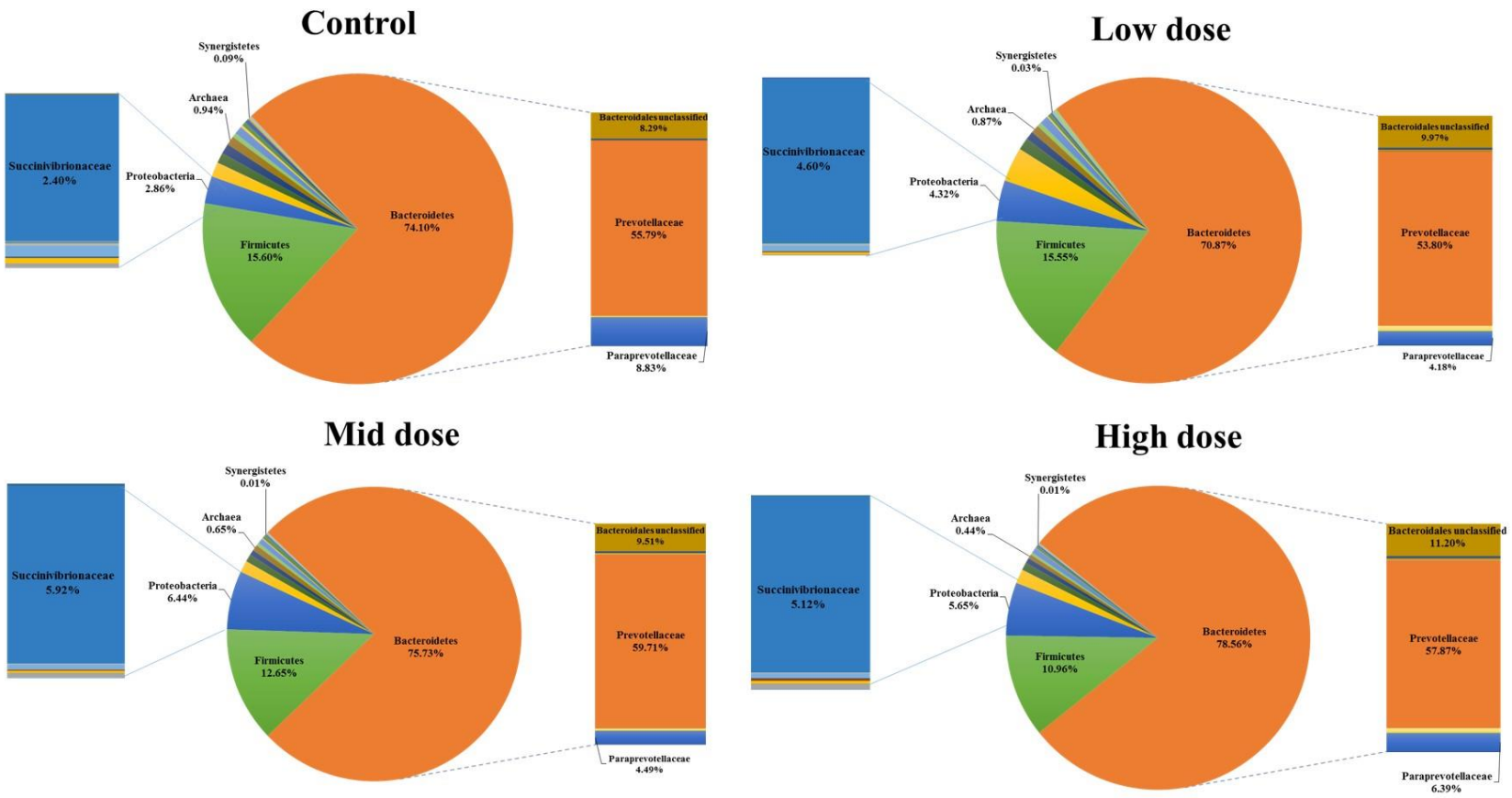


Figure 9. Taxonomic composition of rumen microbiome at the phylum level (pie chart) and family level (bar chart) for control and chloroform (low, mid and high doses) in animals fed with hay:concentrate diet 3 h after feeding.

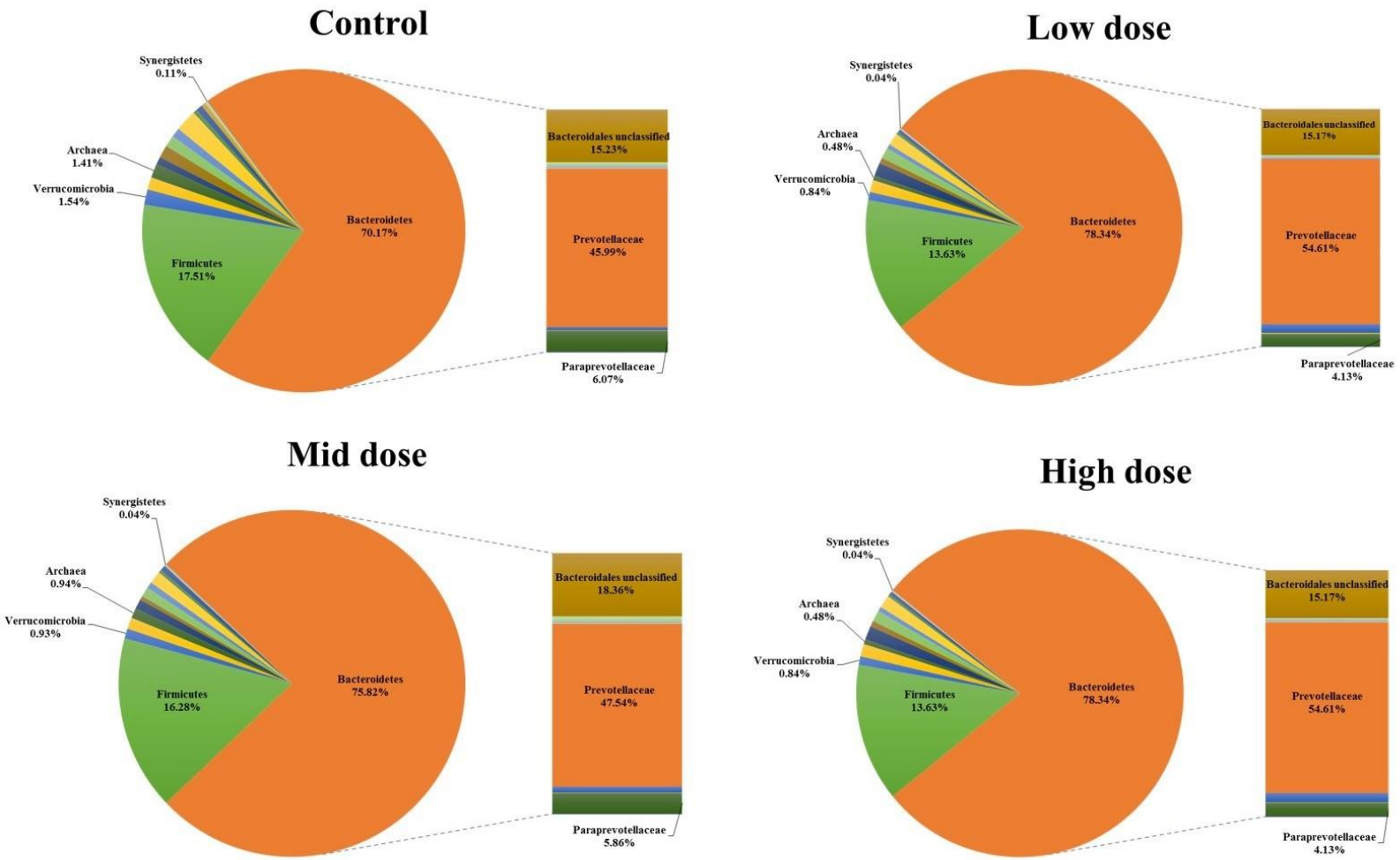


Figure 10. Taxonomic composition of rumen microbiome at the phylum level (pie chart) and family level (bar chart) for control and chloroform (low, mid and high doses) in animals fed with hay diet 3 h after feeding

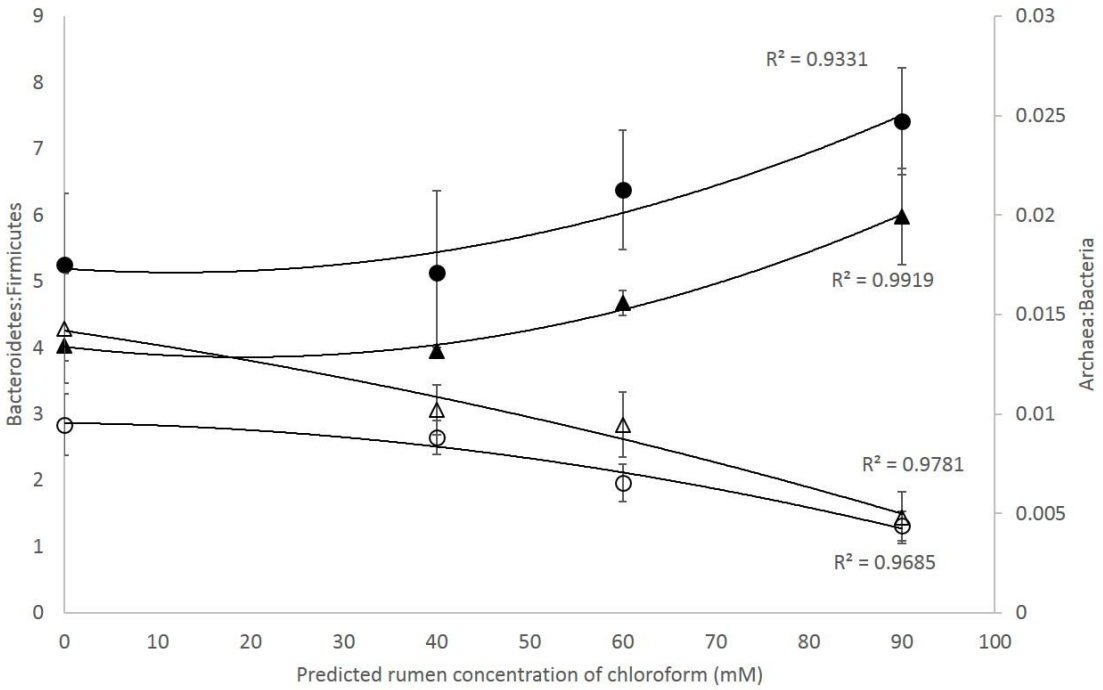


Figure 11. Microbial ratios (Bacteroidetes:Firmicutes (B:F) and Archaea:Bacteria (A:B)) for increasing concentration of chloroform on animals fed with hay (B:F (▲) or A:B (Δ)) or hay:concentrate (B:F (●) or A:B (□)) diet 3 h after feeding. Predicted rumen concentration of chloroform: 40, 60 and 90 µM for low, mid and high dose respectively.

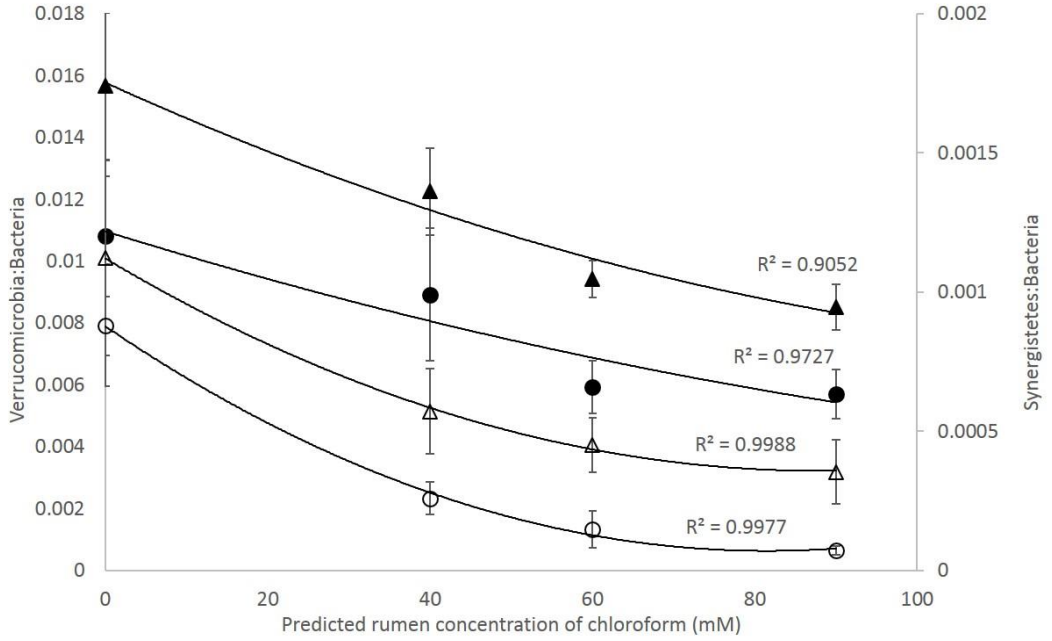


Figure 12. Microbial ratios (Synergistetes:Bacteria (S:B) and Verrucomicrobia:Bacteria (V:B)) for increasing concentration of chloroform on animals fed with hay (V:B (▲) or S:B (Δ)) or hay:concentrate (V:B (●) or S:B (□)) diet 3 h after feeding. Predicted rumen concentration of chloroform: 40, 60 and 90 µM for low, mid and high dose respectively.

Specific OTUs that were significantly increased with chloroform treatment were classified in the *Prevotella* genus, for both diets. Specifically for the animals fed with the hay:concentrate diet, a few OTUs that increased in abundance with chloroform were assigned to Moraxellaceae and Succinivibrionaceae family in the Proteobacteria phylum, and the fibre degrading microorganisms *Fibrobacter succinogenes* and *Ruminoccocus spp*. Regarding the roughage hay fed animals, minor OTUs promoted by the chloroform doses were within the Paraprevotellaceae family and *Butyrivibrio* genus with all the doses and highest dose respectively (Figures 13-18).

The increasing level of chloroform was negatively associated in both diets with the abundance of OTUs assigned to *Prevotella* genus compared with the control, which could suggest a shift within the Prevotella groups through both diets (Figures 13-18). In relation to the fibrolytic microorganisms minor OTUs assigned to *Fibrobacter* genus were decreased in both diets with the lowest dose but did not change or were promoted with the increasing doses of chloroform, which could suggest a shift in those populations. OTUs assigned to the fibrolytic species *R. albus* did not change with the doses, although a single OTU classified as *R. flavefaciens* was suppresed at the highest dose of chloroform with the hay diet.

The Archaea phylum was negatively affected by chloroform levels in both diets in accordance with the decrease observed in the Archaea:Bacteria (A:B) ratio. Specific OTUs assigned to the Methanobacteriaceae family and Methanoplasmatales order were decreased by the chloroform levels.

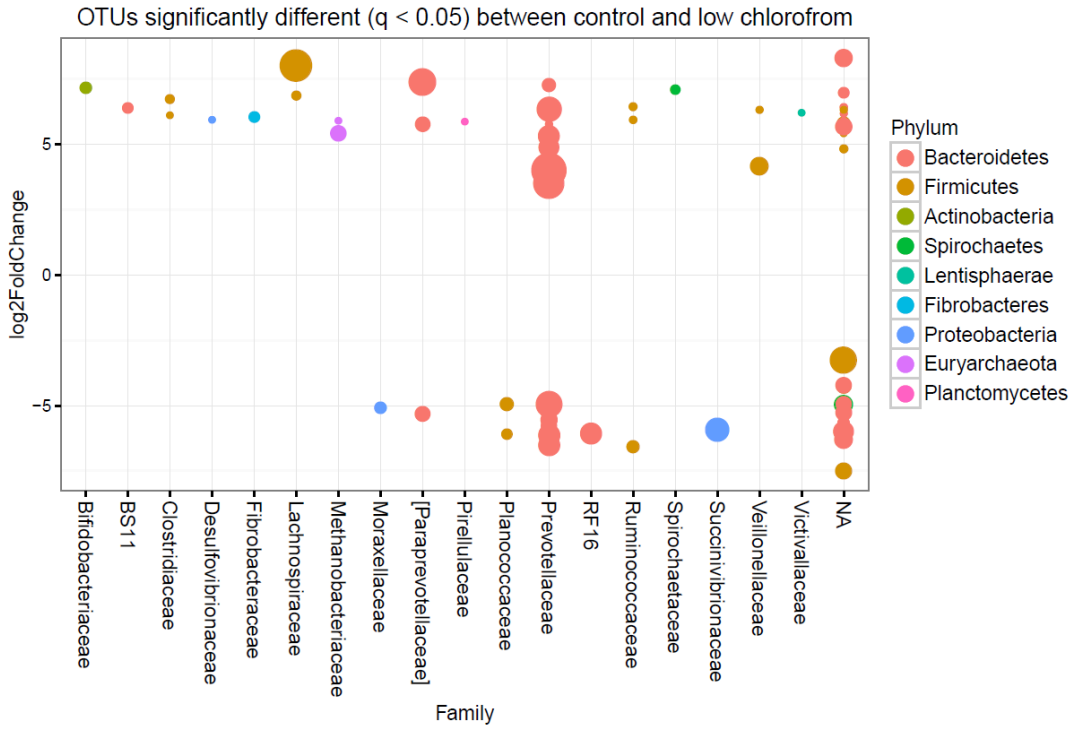


Figure 13. OTUs significantly different (q > 0.05 FDR) at phylum and family level between control and chloroform low dose from animals fed a hay:concetrate diet. Upper axis represents positive fold change of control relative to chloroform treatment while the lower y axis is the negative fold change of the control relative to chloroform low dose. Each point

represents a single OTU colored by phylum, size of point reflects the log2 abundance of the sequence data.

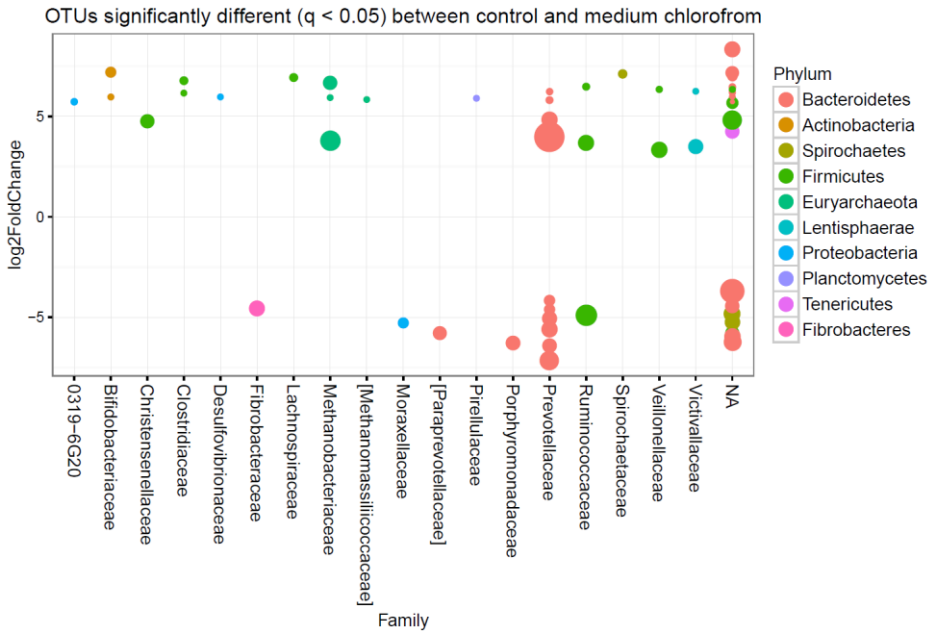


Figure 14. OTUs significantly different (q > 0.05 FDR) at phylum and family level between control and chloroform medium dose from animals fed a hay:concetrate diet. Upper axis represents positive fold change of control relative to chloroform treatment while the lower y axis is the negative fold change of the control relative to chloroform medium dose. Each point represents a single OTU colored by phylum, size of point reflects the log2 abundance of the sequence data.

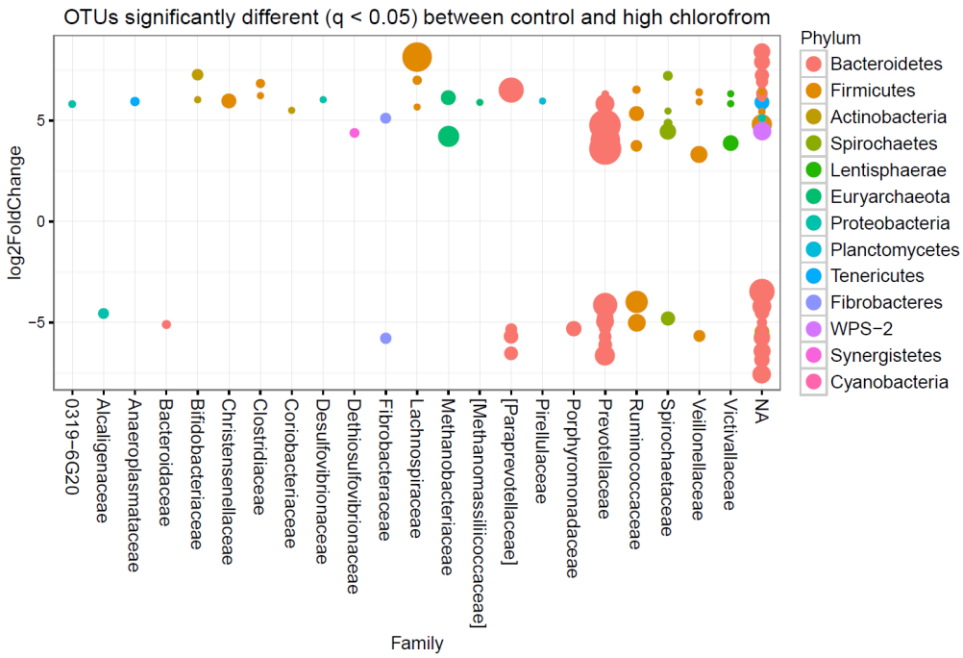


Figure 15. OTUs significantly different (q > 0.05 FDR) at phylum and family level between control and chloroform high dose from animals fed a hay:concetrate diet. Upper axis represents positive fold change of control relative to chloroform treatment while the lower y axis is the negative fold change of the control relative to chloroform high dose. Each point represents a single OTU colored by phylum, size of point reflects the log2 abundance of the sequence data.

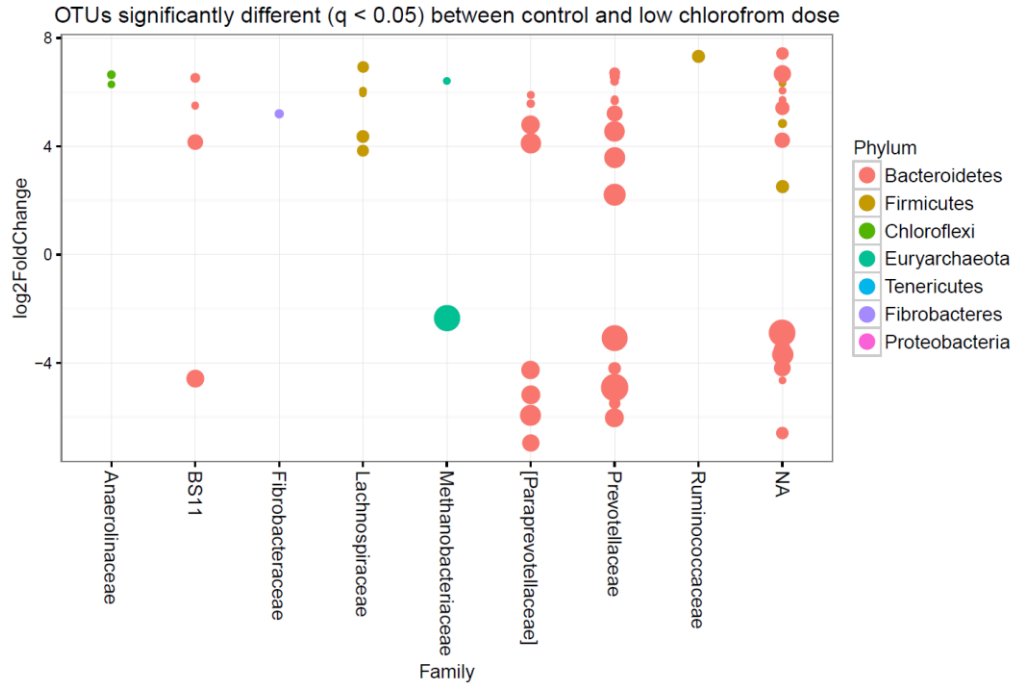


Figure 16. OTUs significantly different (q > 0.05 FDR) at phylum and genus level between control and chloroform low dose from animals fed a hay diet. Upper axis represents positive fold change of control relative to chloroform while the lower y axis is the negative fold change of the control relative to chloroform low dose. Each point represents a single OTU colored by phylum, size of point reflects the log2 abundance of the sequence data.

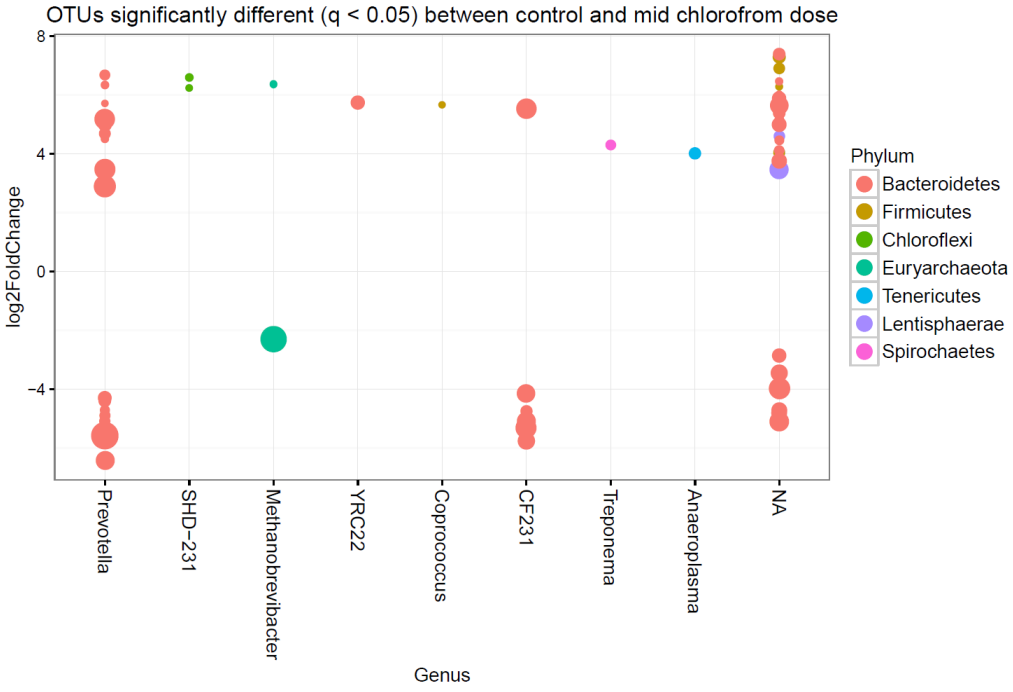


Figure 17. OTUs significantly different (q > 0.05 FDR) at phylum and genus level between control and chloroform mid dose from animals fed a hay diet. Upper axis represents positive fold change of control relative to chloroform while the lower y axis is the negative fold change of the control

relative to chloroform mid dose. Each point represents a single OTU colored by phylum, size of point reflects the log2 abundance of the sequence data.

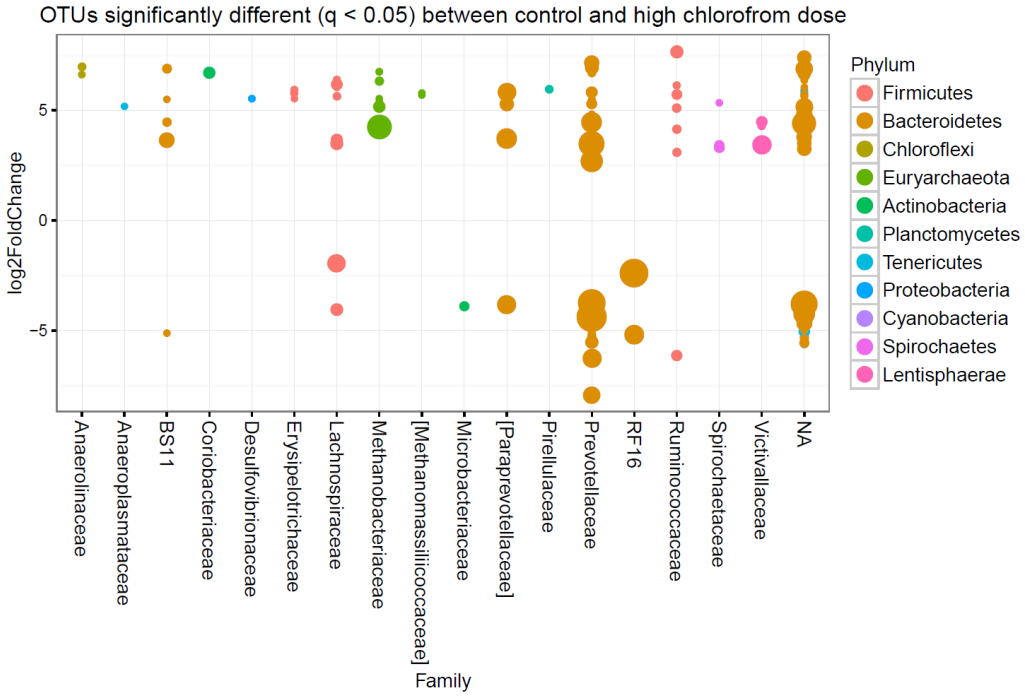


Figure 18. OTUs significantly different (q > 0.05 FDR) at phylum and family level between control and chloroform high dose from animals fed a hay diet. Upper axis represents positive fold change of control relative to chloroform while the lower y axis is the negative fold change of the control relative to chloroform high dose. Each point represents a single OTU colored by phylum, size of point reflects the log2 abundance of the sequence data.

Experiment 2

No significant effects were observed on DMI, methane, hydrogen and fermentation parameters between experimental groups at either the control period or after chloroform treatment for 21 days (Table 8). The daily weight gain was significantly affected by phloroglucinol + chloroform treatment showing an increase compared with the chloroform group and control period.

The methane reduction and hydrogen production were similar to that observed with the medium dose from trial 1. Hydrogen (g) per kg of DMI and the moles of H_2 per mol of CH_4 reduced were significantly decreased ($P \leq 0.05$) in group 1 treated with phloroglucinol + chloroform compared with the group 2 treated with chloroform. The study of the rumen fermentation parameters showed a significant increased ($P = 0.012$) in acetic acid concentration in animals treated with phloroglucinol compared with the positive control group (group 2). The formate concentration increased when methane was reduced as observed in previous trial. However a significantly reduction was observed in animals treated with phloroglucinol + chloroform compared with the chloroform group (group 2).

Non-significant effects were observed in methane, hydrogen and rumen fermentation parameters when experimental animals were inoculated with the acetogenic bacteria (Table 9) compared with control group.

The comparison of the rumen microbiome showed a shift in OTUs numbers (Figure 19) when animals were treated with phloroglucinol + chloroform compared with the chloroform only group. Those OTUs significantly and positively promoted with phloroglucinol treatment were associated to *Prevotella*, *Ruminoccocus*, *Methanobrevibacter* and *Coprococcus*, in which the greater fold change corresponded to *Coprococcus* genus. On the other hand, no effect of the treatments were observed on the abundance of protozoa, FTHFS gene and fungal biomass (Figures 20 and 21).

Table 8. Chloroform-CD (CCD) and Phloroglucinol effects on DMI, methane, hydrogen, daily weight gain and rumen fermentation on animals fed at forage:grain diet.

	Control			CCD 21d			CCD + Phlorog.	CCD		CCD 10 days after treatment		
	Group 1	Group 2	*P-value*	Group 1	Group 2	*P-value*	Group 1	Group 2	*P-value*	Group 1	Group 2	*P-value*
DMI, kg	7.5	7.1	0.50	6.8	6.8	0.95	8.2	7.3	0.23	8.1	7.6	0.40
Methane (g/kg DMI)	22.2	22.3	0.94	13.8	12.6	0.61	11.8	13.8	0.11	15.5	15.3	0.90
Hydrogen (g/kg DMI)	0.13	0.17	0.33	1.15	1.23	0.77	0.44	0.89	0.042	0.36	0.38	0.90
mol H_2/ mol CH_4 reduced*	-	-	-	1.10	1.20	0.79	0.36	0.84	0.006	0.78	0.62	0.75
Daily weight gain, kg	1.00	0.917	0.72	0.988	1.18	0.58	1.38	0.453	0.012	0.130	0.850	0.019
Formate (mM)	0	0	0.99	7.43	5.08	0.69	0.83	11.3	0.046	0.97	9.16	0.111
pH	6.59	6.37	0.33	6.32	6.48	0.32	6.43	6.58	0.27	6.63	6.25	0.071
NH_3-N (mg/100 mL)	9.6	11.7	0.46	12.0	14.9	0.084	14.8	12.9	0.43	14.8	15.5	0.80
Total SCFA, (mM)	83.9	99.8	0.10	94.7	89.9	0.70	94.9	90.4	0.63	85.4	99.3	0.25
SCFA (mol/100 mol)												
Acetate	62.9	68.3	0.16	55.7	56.4	0.74	65.2	60.7	0.012	61.2	60.2	0.51
Propionate	22.2	20.4	0.59	28.5	26.7	0.63	19.4	22.8	0.24	24.8	25.9	0.77
i-Butyrate	0.96	0.80	0.24	0.95	0.75	0.69	0.7	1.1	0.39	0.8	0.6	0.098
Butyrate	11.55	8.37	0.06	11.01	12.20	0.41	11.6	10.7	0.59	10.7	10.8	0.96
i-Valerate	0.99	0.71	0.06	1.04	0.90	0.85	0.9	1.2	0.56	1.0	0.7	0.092
Valerate	0.96	0.98	0.87	1.71	1.93	0.73	1.43	1.60	0.80	0.98	1.07	0.73
Caprionic	0.40	0.49	0.71	1.06	1.12	0.94	0.88	1.42	0.48	0.67	0.78	0.55
A:P	2.95	3.53	0.41	2.08	2.11	0.92	3.46	2.74	0.19	2.52	2.48	0.93

*Taken into account the DMI per animal

Table 9. Chloroform-CD (CCD) and acetogenic bacteria effects on DMI, methane, hydrogen and rumen fermentation parameters from samples collected 3 h after feeding of animals fed with hay:concentrate diet.

	CCD			Acetogen + CCD	CCD		CCD 14 days after treatment		
	Group 1	Group 2	*P-value*	Group 1	Group 2	*P-value*	Group 1	Group 2	*P-value*
DMI, kg	8.1	7.6	0.42	8.0	7.7	0.54	8.0	7.9	0.83
Methane (g/kg DMI)	14.7	16.1	0.45	15.7	17.3	0.35	16.0	17.2	0.36
Hydrogen (g/kg DMI)	0.33	0.41	0.58	0.39	0.38	0.95	0.46	0.40	0.74
pH	6.33	6.57	0.29	6.54	6.64	0.55	6.23	6.47	0.28
Total SCFA, (mM)	89.9	94.8	0.70	97.3	93.6	0.725	109.2	103.7	0.66
Fatty acid (mol/100 mol)									
Acetate	60.7	60.7	0.99	63.4	65.6	0.43	59.3	63.0	0.043
Propionate	25.7	25.1	0.88	21.8	20.4	0.60	25.0	23.0	0.34
i-Butyrate	0.74	0.63	0.28	0.62	0.99	0.08	0.84	0.83	0.97
Butyrate	10.4	11.0	0.80	11.2	9.6	0.29	11.1	9.7	0.18
i-Valerate	0.90	0.75	0.36	0.63	1.13	0.16	0.90	0.90	0.99
Valerate	0.94	1.11	0.51	1.51	1.34	0.66	1.64	1.60	0.93
Caprionic	0.68	0.77	0.64	0.82	1.03	0.59	1.26	1.04	0.65
A:P	2.40	2.60	0.69	3.03	3.25	0.63	2.42	2.76	0.24

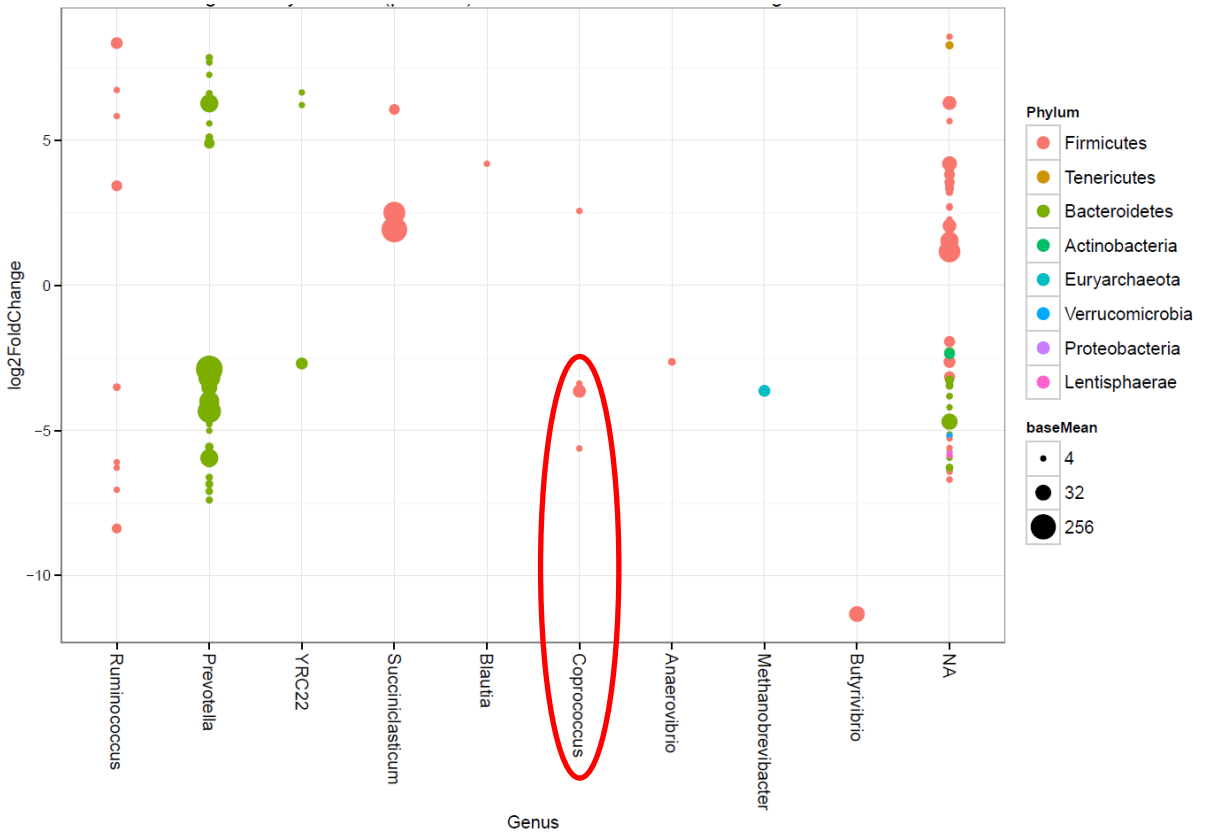


Figure 19. OTUs significantly different (q > 0.05 FDR) at phylum and genus level between chloroform (Group 2) and Phloroglucinol treatment (Group 1) at day 37. Upper axis represents positive fold change of chloroform (Group 2) relative to phloroglucinol (Group 1) while the lower y axis is the negative fold change of the chloroform (Group 2) relative to phloroglucinol (Group 1). Each point represents a single OTU coloured by phylum, size of point reflects the log2 abundance of the sequence data.

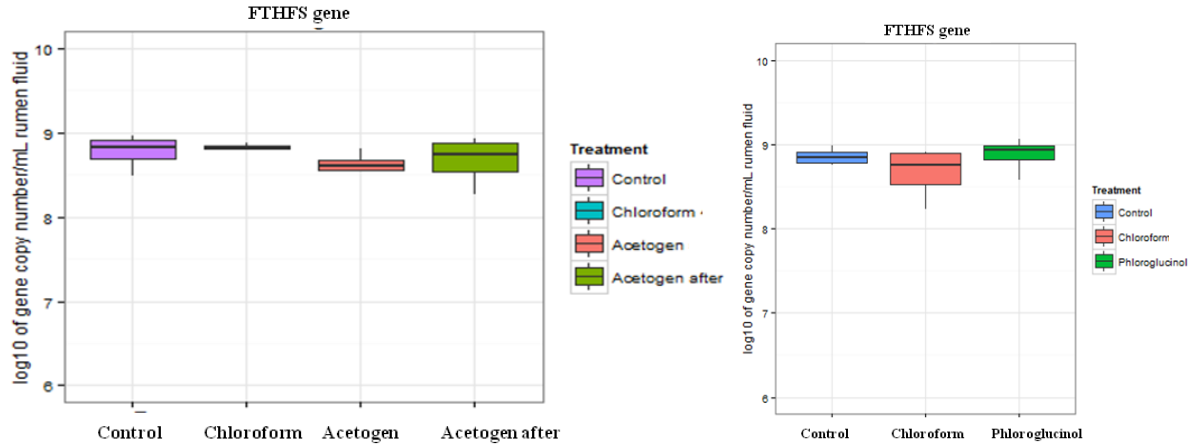


Figure 20. Quantitative PCR analysis of FTHFS gene changes in response to chloroform, phloroglucinol and acetogenic bacteria.

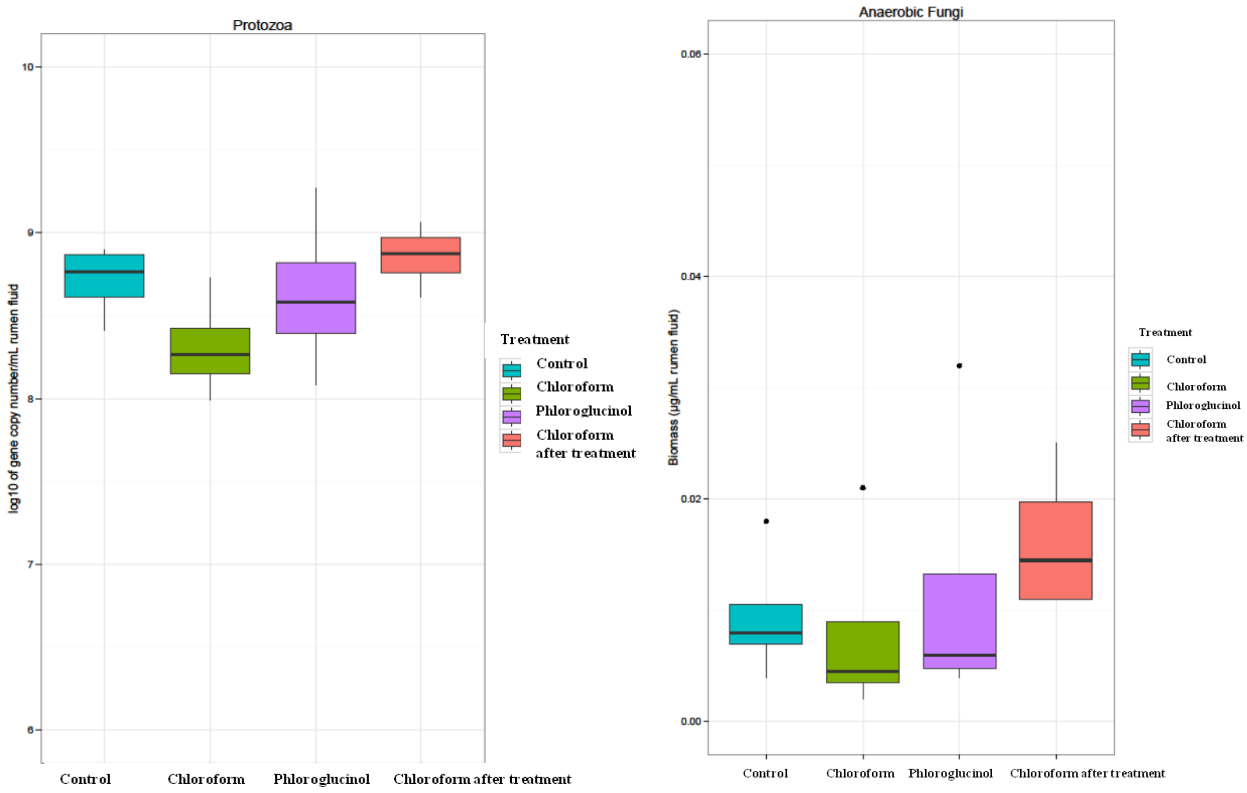


Figure 21. Quantitative PCR analysis of protozoa and anaerobic fungi population changes in response to pre-control, chloroform and phloroglucinol.

Experiment 3

No significant effects were observed on DMI, methane, hydrogen and fermentation parameters between groups at control period and chloroform treatment after 21 days (Table 10). Dry matter intakes were significantly (P = 0.026) increased in animals treated with chloroform and previously treated with phloroglucinol compared with animals that only received chloroform. The methane reduction was similar to that observed with the medium dose from trial 1 and 2. Hydrogen expelled from the animals was not detected during the experiment which is in contrast with previous trials using chloroform which observed an increase in hydrogen production when methane was decreased at a similar extent (Martinez-Fernandez et al. 2014; "In: Proceedings of the 30th Biennial Conference of the Australian Society for Animal Production"). A significant (P ≤ 0.05) increased was observed in total SCFA, acetic acid concentration and acetic:propionic ratio in animals treated with phloroglucinol compared with chloroform group. The daily weight gain was significantly affected by phloroglucinol + chloroform treatment showing an increase compared with the chloroform group and control period.

The ruminal degradation of DM of Rhode grass hay assessed in sacco showed no significant effects of chloroform and phloroglucinol treatments on rumen degradability after 24 h, although a tendency was observed for reduced rumen degradability after 48 h with the treatment groups compared with the pre and post control periods (Table 11).

The dose effect of chloroform on the abundance of FTHFS gene, protozoa and fungal biomass are shown in figures 23 and 24. No effect was observed on FTHFS gene abundance, and the abundance of protozoa only increased significantly with chloroform treatment compared with the post treatment period. Anaerobic fungi biomass was significantly affected (P ≤ 0.05) by phloroglucinol treatment, showing an increase compared with the chloroform treated animals.

Table 10. Chloroform-CD (CCD) and phloroglucinol effects on DMI, methane and hydrogen production, daily weight gain and rumen fermentation parameters from samples collected 3 h after feeding of animals fed at forage diet.

	Control			CCD 21 days			CCD + Phloroglucinol	CCD		CCD 14 days after Phloroglucinol		
	Group 1	Group 2	*P-value*	Group 1	Group 2	*P-value*	Group 1	Group 2	*P-value*	Group 1	Group 2	*P-value*
DMI, kg	7.6	7.0	0.35	7.1	6.6	0.17	6.8	6.4	0.18	7.8	7.1	0.026
Methane (g/kg DMI)	24.4	23.2	0.18	16.2	16.2	0.93	17.1	19.9	0.100	19.8	18.5	0.22
Hydrogen (g/kg DMI)	0.00	0.00	-	0.00	0.00	-	0.00	0.00	-	0.00	0.00	-
Daily weight gain, kg	0.233	0.183	0.005	0.119	-0.059	0.31	0.571	-0.012	0.009	0.500	0.732	0.11
NH_3-N (mg/100 mL)	9.4	8.8	0.57	24.9	28.1	0.41	23.9	28.1	0.12	32.6	39.1	0.12
Total SCFA, (mM)	94.8	94.7	0.98	89.9	94.7	0.40	104.6	88.8	0.003	85.7	94.9	0.002
SCFA (mol/100 mol)												
Acetate	67.2	70.0	0.18	73.8	74.1	0.85	80.8	75.3	0.002	75.5	76.6	0.29
Propionate	16.0	16.6	0.27	16.7	16.3	0.71	11.3	15.8	0.001	15.0	15.7	0.031
i-Butyrate	1.4	0.6	0.068	1.1	1.3	0.28	0.9	1.3	0.003	1.3	1.2	0.029
Butyrate	9.6	9.6	0.95	5.0	4.9	0.85	4.7	4.7	0.99	5.0	3.5	0.11
i-Valerate	1.8	1.1	0.11	1.7	1.7	0.81	1.1	1.4	0.008	1.5	1.3	0.063
Valerate	2.4	1.4	0.055	1.4	1.4	0.62	0.9	1.2	0.003	1.38	1.43	0.64
Caprionic	1.6	0.7	0.057	0.3	0.3	0.60	0.2	0.2	0.17	0.22	0.19	0.18
A:P	4.2	4.2	0.87	4.5	4.5	0.81	7.2	4.8	0.001	5.04	4.88	0.26

Table 11. Chloroform-CD and phloroglucinol effects on DM degradation of hay at 24 and 48 h

	Control	Chloroform 21 days	Phloroglucinol 42 days	Post treatment	*P-Value*
DM degradability Hay % (24 h)	33.5	31.8	33.1	33.8	0.124
DM degradability Hay % (48 h)	39.8	39.3	39.0	40.9	0.058

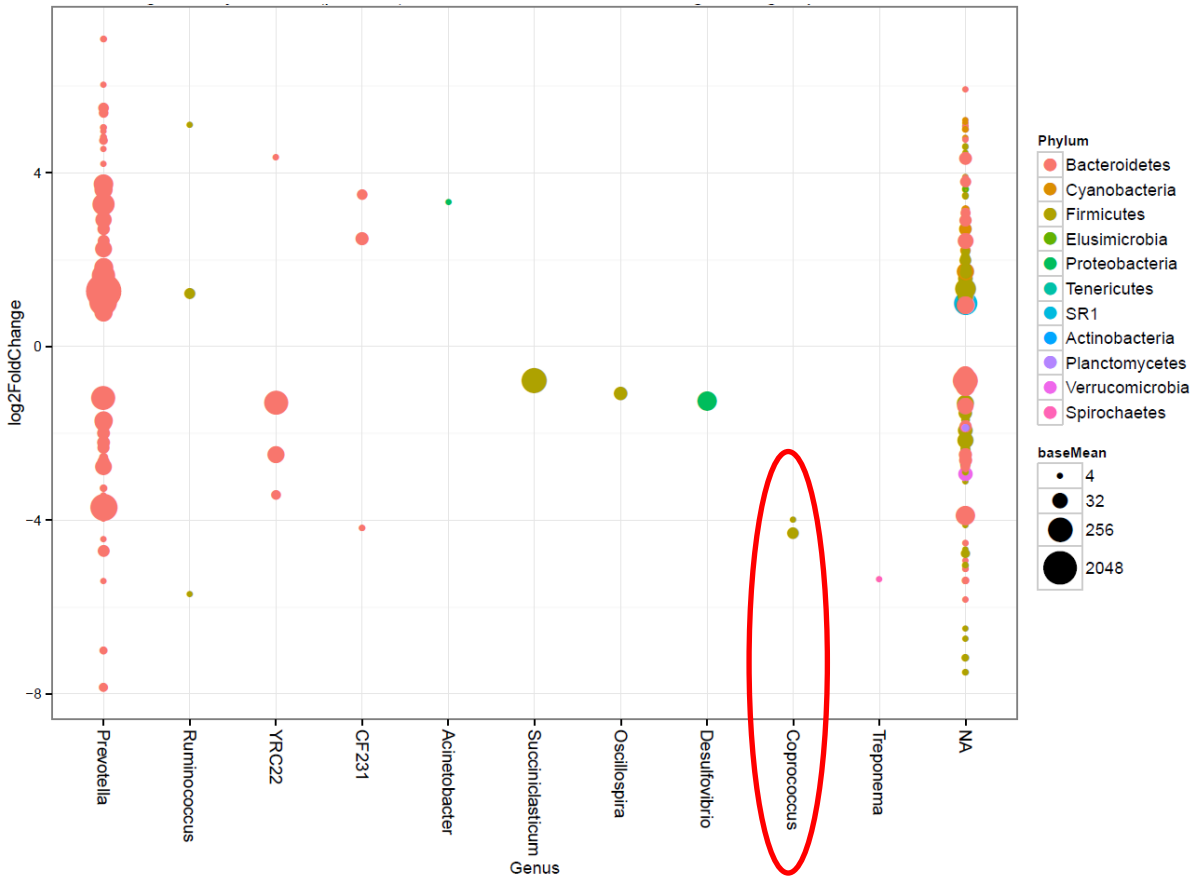


Figure 22. OTUs significantly different (q > 0.05 FDR) at phylum and genus level between chloroform (Group 2) and Phloroglucinol treatment (Group 1) at day 42. Upper axis represents positive fold change of chloroform (Group 2) relative to phloroglucinol (Group 1) while the lower y axis is the negative fold change of the chloroform (Group 2) relative to phloroglucinol (Group 1). Each point represents a single OTU coloured by phylum, size of point reflects the log2 abundance of the sequence data.

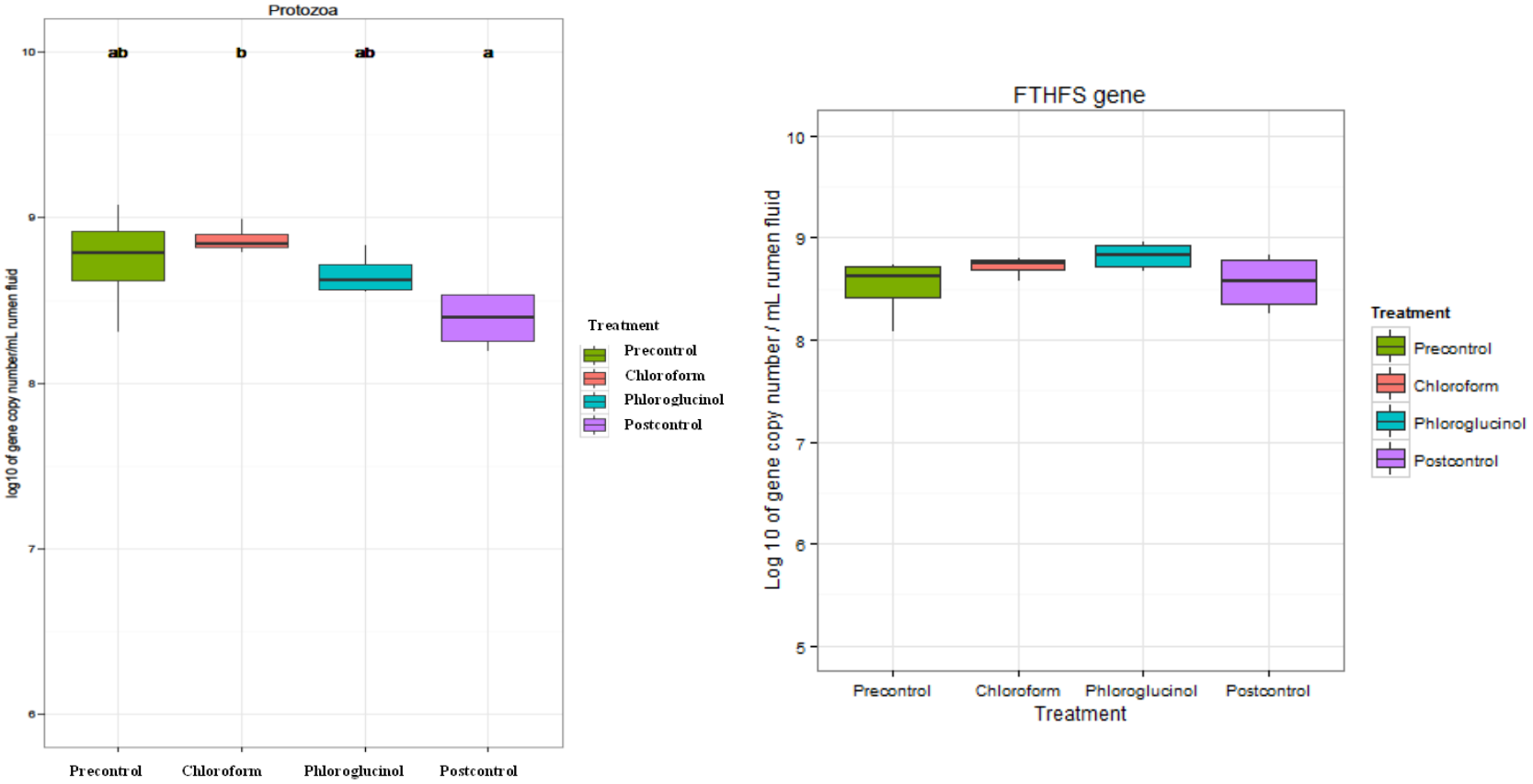


Figure 23. Quantitative PCR analysis of FTHFS gene and protozoa changes in response to chloroform and phloroglucinol in animals fed with forage diet. [a,b] Letters denote significant differences between treatments, bars that do not share the same letter for a species are significantly different for each other (P< 0. 05).

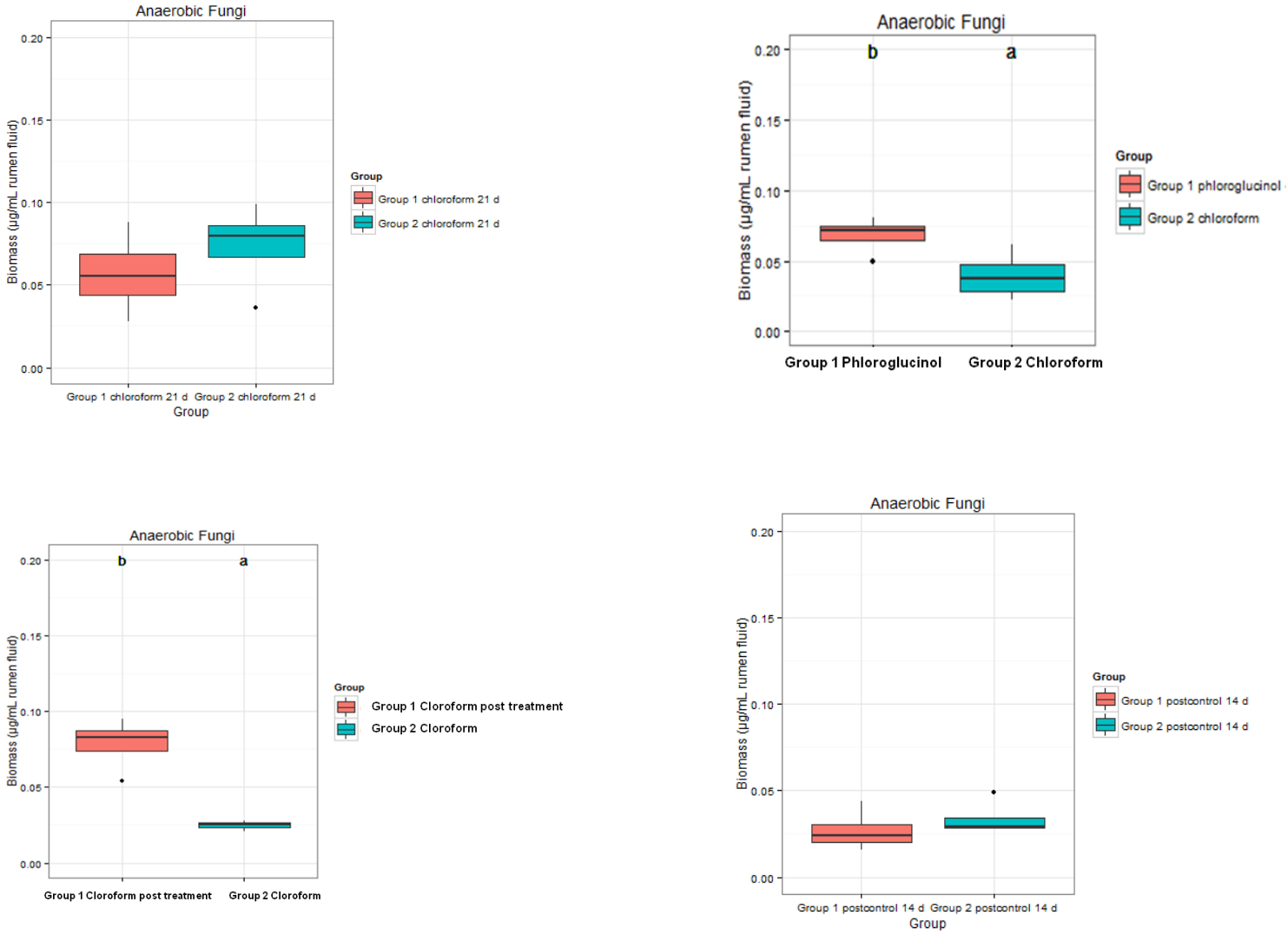


Figure 24. Quantitative PCR analysis of anaerobic fungi population changes between groups. [a,b] Letters denote significant differences between treatments, bars that do not share the same letter for a species are significantly different for each other ($P < 0.05$).

Experiment 4

Forage diet

No significant effects were observed on DMI, methane and hydrogen between animals groups at control period with the hay diet (**Table 12**). A significant increase in ammonia concentration was observed in those animals treated with urea or casein compared with the animals fed just with hay. Dry matter intakes were significantly ($P \leq 0.001$) decreased in animals treated with chloroform without additive or urea than animals treated with chloroform plus casein. A 90% reduction of CH_4 (g) per kg of DMI was observed with the chloroform treatment through the 3 groups compared with the control period. Hydrogen expelled from the animals fed with hay and treated with chloroform and casein was lower than the other 2 groups although it was not significant. The study of the rumen fermentation parameters showed a significant reduction ($P = 0.018$) of ammonia in animals treated with chloroform without additive or urea compared with the chloroform plus casein group. A significant increase in butyrate and i-butyrate was observed in animals treated with chloroform and casein. During the control period the 3 treatments (no additive, urea and casein) showed a similar daily weight lost, however when animals were treated with chloroform a trend was observed, showing the casein additive a lower daily weight lost than the other treatments and control period. Animals treated with chloroform without additive showed a significant reduction on DM rumen degradability after 24 and 48 h compared with the animals which received urea or casein (Table 12).

Forage:concentrate diet

No significant effects were observed on DMI, methane and hydrogen between groups at control period with the hay:concentrate diet (**Table 13**). A significant increase in ammonia concentration was observed in those animals treated with urea or casein compared with the animals fed just with hay:concentrate. Dry matter intakes were significantly ($P \leq 0.001$) decreased in animals treated with chloroform without additive or casein than animals treated with chloroform plus urea. A range of 65-50% reduction of CH_4 (g) per kg of DMI was observed was observed with the chloroform treatment through the 3 groups compared with the control period. Hydrogen (g) per DMI expelled from the animals fed with hay:concentrate and treated with urea was significantly lower than the other 2 groups. However, the moles of H_2 per mol of CH_4 reduced were not significantly affected. The study of the rumen fermentation parameters showed a significant reduction ($P = 0.018$) of ammonia in animals treated with chloroform without additive compared with the groups treated with chloroform plus casein or urea. Although total SCFA were not significantly affected, a greater concentration was observed in animals treated with chloroform plus urea. During the control period, the 3 treatments (no additive, urea and casein) showed a similar daily weight lost. However, when animals were treated with chloroform was observed a positive increase, particularly with casein and urea treatments. No significant differences were observed on rumen hay or concentrate degradability after 24 and 48 h (Table 12).

Table 12. Control and Chloroform-CD effects on DMI, CH_4 and H_2 production, daily weight gain and rumen fermentation parameters from samples collected 3 h after feeding of animals fed at forage diet without feed additive, urea or casein.

	Control				Chloroform-CD 21 days			
	Hay	Hay+ Urea	Hay + Casein	*P-value*	Hay	Hay+ Urea	Hay + Casein	*P-value*
DMI, kg	5.0	5.1	5.3	0.93	3.2^{c}	4.2^{b}	5.2^{a}	0.001
Methane (g/kg DMI)	20	20	18	0.27	0.17	1.12	2.99	0.24
Hydrogen (g/kg DMI)	0.00	0.00	0.00	-	1.26	1.09	0.75	0.45
mol H_2/ mol CH_4 reduced*	-	-	-	-	0.51	0.45	0.36	0.746
24 h hay in saco DM degradabiliy %	21	21	19	0.30	13^{b}	18^{a}	17^{a}	0.027
48 h hay in saco DM degradabiliy %	28	29	27	0.33	20^{b}	23^{a}	25^{a}	0.018
Daily weight gain, kg	-1.24	-1.00	-1.17	0.61	-0.840	-0.698	-0.127	0.054
pH	6.7	6.9	6.9	0.31	7.0	6.9	6.9	0.74
Formate mM	0	0	0	-	13.5	11.7	8.8	0.39
NH_3-N (mg/100 mL)	9.8^{b}	20.0^{a}	27.6^{a}	0.011	3.2^{c}	9.4^{b}	19.2^{a}	0.001
Total SCFA, (mM)	55.9	60.3	61.4	0.61	38.8	42.7	52.9	0.17
Fatty acid (mol/100 mol)								
Acetate	70.4	72.3	71.1	0.17	54.6	58.7	55.2	0.58
Propionate	18.3	17.9	17.0	0.12	28.7	25.8	23.7	0.15
i-Butyrate	1.1^{b}	0.89^{b}	1.8^{a}	0.018	1.3^{b}	0.94^{b}	2.4^{a}	0.014
Butyrate	7.1^{a}	6.2^{ab}	5.4^{b}	0.011	7.3	8.3	8.9	0.063
i-Valerate	1.5^{b}	1.3^{b}	2.4^{a}	0.002	5.4	3.9	5.6	0.36
Valerate	1.1^{b}	1.0^{b}	1.9^{a}	0.005	2.0	1.8	3.5	0.14
Caprionic	0.5	0.4	0.4	0.95	0.7	0.6	0.8	0.90
A:P	3.9	4.0	4.2	0.18	1.9	2.3	2.4	0.46

*Taken into account the DMI per animal

$^{a–c}$Within a row treatment means for each diet without a common superscript differ, $P < 0.05$.

Table 13. Control and Chloroform-CD effects on DMI, CH_4 and H_2 production, daily weight gain and rumen fermentation parameters from samples collected 3 h after feeding of animals fed at forage:concentrate diet without feed additive, urea or casein.

	Control				Chloroform-CD 21 days			
	Grain	Grain + Urea	Grain + Casein	*P-value*	Grain	Grain + Urea	Grain + Casein	*P-value*
DMI, kg	7.4	7.5	7.8	0.76	3.2^{b}	7.1^{a}	6.5^{ab}	0.023
Methane (g/kg DMI)	20.0	20.1	19.5	0.96	5.8	11.2	9.4	0.37
Hydrogen (g/kg DMI)	0.00	0.00	0.00	0.57	2.6^{a}	1.0^{b}	2.4^{a}	0.019
mol H_2/ mol CH_4 reduced*	-	-	-	-	1.60	1.16	1.93	0.514
Daily weight gain, kg	-0.238	-0.119	-1.23	0.28	0.260	0.730	0.731	0.25
24 h hay in saco degradabiliy %	21	24	20	0.31	21	20	16	0.29
48 h hay in saco degradabiliy %	31	35	29	0.25	31	31	28	0.17
24 h grain in saco degradabiliy %	53	52	53	0.82	53^{a}	51^{ab}	50^{b}	0.045
48 h grain in saco degradabiliy %	55	56	55	0.82	57	56	54	0.11
pH	6.6	6.5	6.5	0.77	7.1^{a}	6.8^{ab}	6.6^{b}	0.038
Formate mM	2.0	2.8	2.5	0.87	20.1	18.7	18.4	0.87
NH_3-N (mg/100 mL)	8.0^{b}	19.0^{a}	25.7^{a}	0.012	10.6^{c}	15.6^{b}	21.4^{a}	0.004
Total SCFA, (mM)	75.9	88.3	83.6	0.58	43.5	84.2	62.2	0.14
Fatty acid (mol/100 mol)								
Acetate	64.9^{a}	67.1^{a}	61.5^{b}	0.007	51.8	55.1	48.4	0.073
Propionate	18.0	18.1	21.6	0.18	23.7	25.1	23.8	0.82
i-Butyrate	0.88^{b}	0.80^{b}	1.46^{a}	0.015	1.8	0.9	2.2	0.17
Butyrate	12.5	10.7	10.4	0.41	14.5	13.6	16.7	0.58
i-Valerate	2.0	1.8	2.5	0.100	5.1	2.8	4.6	0.38
Valerate	1.5^{b}	1.3^{b}	2.5^{a}	0.005	2.2^{ab}	1.9^{b}	3.3^{a}	0.048
Caprionic	0.20	0.17	0.14	0.37	0.8	0.6	0.9	0.65
A:P	3.6^{a}	3.7^{a}	2.9^{b}	0.048	2.2	2.2	2.1	0.73

*Taken into account the DMI per animal

$^{a–c}$Within a row treatment means for each diet without a common superscript differ, $P < 0.05$.

4. Discussion

The first trial established a model in cattle whereby methanogens were directly inhibited in a dose dependant manner and the subsequent responses in rumen microbial metabolism were evaluated. The three levels of chloroform, low, medium and high, reduced CH_4 production by approximately 14, 37 and 55%, respectively with both diets compared to the control period with no apparent effect on feed intakes. Conversely H_2 expelled by treated animals showed a dose-dependent increase as CH_4 decreased. A similar inverse relationship between CH_4 reduction and H_2 loss has been reported in dairy cows treated with nitrate or 3-nitrooxypropanol, and goats fed the CH_4 inhibitor BCM (Veneman et al., 2015, Mitsumori et al., 2012, Hristov et al., 2015). However, importantly this study showed that greater amounts of H_2 (1.7 – 2.9 fold; g/kg DMI) were expelled in animals supplemented with the hay:concentrate diet compared to the hay only diet. Furthermore the amount of H_2 expelled in the CH_4 inhibited animals was lower than the predicted amount of H_2 involved in hydrogenotrophic CH_4 formation (4 moles of H_2/mole CH_4). This suggests that significant amounts of H_2 were redirected into reduced end products and perhaps microbial protein. Interestingly the rumen microbiota in the roughage hay fed animals appeared to utilise more H_2 that was available from the reduction in CH_4 formation than the hay:concentrate fed cattle, possibly due to the slower fermentation rate of the hay diet compare with the highly fermentable concentrate diet. These observations are further supported by the metabolomic data which showed an increase in more reduced metabolites and greater nitrogen availability particularly in the roughage hay fed animals.

In relation to the redirection of H_2, on both diets there was a shift in fermentation from acetate to fatty acids that were longer in length particularly propionate which 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 the halogenated methane analogue, BCM, as the methane inhibitor (Mitsumori et al., 2012; Abecia et al., 2012; Denman et al., 2007). The rumen microbiome analysis showed specific OTUs assigned to the *Prevotella* genus were promoted when methanogenesis was inhibited. Some of these *Prevotella* OTUs promoted by chloroform with the hay:concentrate diet, were closely associated with the *Prevotella* group 7 which was increased in goats inhibited with BCM (Mitsumori et al., 2012). *Prevotella* species appear to increase propionate production via the randomizing pathway when methanogenesis is inhibited (Denman et al., 2015). The *Prevotella* OTUs, promoted in the chloroform treated animals, may occupy the niche vacated by those *Prevotella* OTUs that declined as a result of increasing chloroform concentration. OTUs assigned to the *Butyrivibrio* genus were positively affected by chloroform in roughage fed animals, which is a likely contributor to the increased butyrate in these animals.

Another potential H_2 sink in absence of methanogenesis is acetate produced from reductive acetogenesis (Fonty et al., 2007). In the present study the acetic acid concentration decreased with chloroform treatments, which possibly indicates that reductive acetogenic bacteria have not contributed significantly to the redirection of H_2. However, it has been observed that chloroform might inhibit acetogens (Knight et al., 2011). The notion that acetogens would be promoted through an increase in availability of metabolic H_2 in the

methane inhibited rumen remains unresolved and may be confounded by using chemical inhibitors that target methanogenesis.

A particularly interesting observation was the gradual increase in formate concentration as methane formation declined and hydrogen accumulated. This has been observed previously when CH_4 analogues such as chloroform inhibited methanogenesis (Thiele and Zeikus, 1988). A meta-analysis of studies involving methane inhibition also identified that increased formate was a characteristic response to methane inhibition Ungerfeld (2015). Leng (2014) suggested that formate accumulates when methanogensis is inhibited, and this helps to maintain a steady partial pressure of metabolic hydrogen in the rumen fluid. Formate is produced by ruminal bacteria and fungi and it is mainly consumed by specific methanogens as a precursor for CH_4 formation (Hungate et al., 1970, Asanuma et al., 1998, Bauchop and Mountfort, 1981). Leng (2014) cites studies showing that some methanogens can produce formate when inhibited with halogenated hydrocarbons. It is possible therefore that the accumulation of formate might be due to a balance between an increase in production and a decrease in utilisation, when methanogenesis is inhibited. Formate might help in the control of metabolic hydrogen partial pressures in the rumen, playing a role as hydrogen sink and being an indicator of H_2 partial pressure. This is in accord with the present study where methane inhibited animals fed with the hay:concentrate diet had higher concentrations formate and H_2 release than those only fed with the roughage hay.

A shift in SCFA pattern and increase in formate were not the only significant changes in microbial end products as H_2 concentration rose. There were increases in amino acids, ammonia and nucleic acids, which could be indicative of an increase in proteolysis and microbial growth. In our study, metabolites (such as hypoxanthine, inosine or nicotinic acid) which are degradation products of microbial cells, increased with chloroform treatment. Nucleic acids can breakdown into inosine, xanthine, hypoxanthine, and uracil, and nicotinic acid can increase microbial protein synthesis (McAllan and Smith, 1973, Riddell et al., 1980). The amino acid profile showed an increase in valine, leucine and isoleucine that could be due to greater digestion of protein in the rumen. Increases in those amino acids and SCFA have been observed as previously mentioned. Another important amino acids which increased when methanogenesis was inhibited, is aspartate. This amino acid is the transamination product of oxaloacetic acid which is produced in the succinate–propionate (randomizing) pathway that is considered as a major route for propionate synthesis in the rumen (Baldwin et al., 1963, Joyner and Baldwin, 1966) and might suggest an energy redirection. An abundance of genes assigned to this pathway were found to be increased and predominately associated with *Prevotella* species in goats administered with the anti-methanogenic BCM (Denman et al., 2105). Other intermediates of the randomizing pathway, such as malate and fumarate, increased when methanogenesis was inhibited particularly with the roughage diet, supporting the redirection found towards a more propionic acid production. The increase in amino acids in the rumen may be due to proteolytic activity associated with the relative increase in Bacteroidetes and *Prevotella* related bacteria. Ungerfeld (2015) suggested that inhibition of methanogens, could stimulate amino acids and fatty acids synthesis and therefore the increase in microbial biomass would also be a H_2 sink under these conditions.

The high Bacteroidetes:Firmicutes (B:F) ratio (~75:17) observed in the untreated animals for both diets was substantially higher than has been reported in many other ruminant studies where temperate diets are common. Our study involving tropical adapted cattle fed a basal diet of tropical hay is in agreement with the high ratio reported by McCann et al., (2014) in Brahman cattle fed Coastal Bermuda-grass. In this study, a further increase in this ratio was observed in both diets when methane was decreased and is consistent with the redirection of hydrogen in the rumen. The Bacteroidetes are considered net H_2 utilisers whereas the Firmicutes phylum contains a higher number of H_2 producers (Stewart et al., 1997). The same B:F shift was observed when methane was reduced *in vivo* and *in vit*ro when methanogenesis was inhibited with BCM (Denman et al., 2015, Martinez-Fernandez et al., 2015). Cattle fed concentrate, had a significant population of Proteobacteria (~ 3%) compared with the roughage hay only diet and within this phylum, the predominant family Succinivibrionaceae which are involved in propionate production, increased with the three levels of chloroform. A higher abundance of this family in low-emission beef cattle compared with high emitters has also been observed (Wallace et al., 2015). Wallace et al. (2015) hypothesized that metabolic hydrogen could be used by these bacteria to produce succinate, so they may be key bacteria in the redirection of H_2 in animals fed concentrate/ high quality diets.

Bacteria affiliated with the Synergistetes and Verrucomicrobia phyla were negatively correlated with the increasing levels of chloroform and increases in H_2, for both diets. A similar negative relationship for these groups of bacteria and increased rumen H_2 has been previously reported in ruminants treated with BCM (Denman et al., 2015). In high and low methane emitting animals, Synergistetes were significantly more abundant in high emitters and there was a tendency for Verrumicrobia to also be higher in these animals as well (Wallace et al., 2015). Collectively these data may indicate that Synergistetes and Verrucomicrobia bacteria are sensitive indicators of H_2 partial pressures in the rumen fluid. Recently, Leong et al. (2016) demonstrated that interspecies H_2 transfer between Synergistetes and methanogens enhanced the growth of the bacterium which may explain why these bacteria declined in abundance when methanogens were inhibited in the current study.

In relation to the rumen archaeal community, the A:B ratio decreased with increasing levels of chloroform, which represented a 5-7 fold decrease in the methanogen population and a 30-50% decrease in methane production. Furthermore the rumen microbiome analysis revealed that OTUs assigned to Methanobacteriaceae family and Methanoplasmatales order decreased with the increasing doses of chloroform. Our results are in agreement with previous studies which observed a similar decrease using halogenated compounds to inhibit enteric methanogenesis in cattle (Denman et al., 2007). Mitsumori et al. (2012) observed that only a half-log reduction in the methanogen population was correlated with 50% reduction in methane when BCM was used. Previous studies (Wallace et al., 2015, Roehe et al., 2016) have also reported a higher proportion of A:B ratio from high methane emitting animals compared with low emitters. The decreasing A:B ratio observed when methanogenesis was inhibited may provide a simple index for relative methane production in ruminants.

It is assumed that H_2 accumulation in the rumen impairs fibre digestion and therefore would reduce productivity (Wolin et al., 1997, Janssen, 2010). However a previous study showed that inhibition of methanogenesis by BCM in small ruminants dramatically increased H_2 without affecting DMI and feed digestibility (Mitsumori et al., 2012). In the present study, dry matter intakes were not affected and OTUs assigned to the fibrolytic species *R. albus* and *R. flavefaciens* did not change through the chloroform low and mid doses compared with control period. Importantly, OTUs classified as *F. succinogenes* were positively associated with chloroform in the hay:concentrate diet and were only negatively associated with the lowest dose in both diets. It is known that this species is not affected by hydrogen accumulation and does not produce it in the rumen. Our results suggest that the fibrolytic population was not affected by the increasing level of H_2, although the relative abundance of some particular OTUs changed. Further analyses to study how those changes affect the fibre degradability in the rumen should be carried out in future experiments.

Protozoa and fungi also play a key role in fibre degradation and rumen metabolism, and can be affected by the H_2 accumulation in the rumen. No significant changes were observed in the abundance of those populations suggesting a non-detrimental effect on fibre digestion and proteolysis, supported as well by the metabolite and fermentation profiles discussed previously. However, further analyses using in-depth sequencing technology could be developed in future experiments to understand these important rumen community members.

The second *in vivo* experiment studied the availability of supplements and microbial treatments to consume hydrogen when methane was reduced. Promising results with one of the supplements were obtained regarding the hydrogen redirection in the rumen. The results showed a decrease of free hydrogen gas when methanogenesis was inhibited in animals treated with phloroglucinol compared with the positive control group (only treated with chloroform). A shift towards an increase of acetic acid concentration was observed in those animals treated with phloroglucinol. Our results suggest a redirection of the excess of hydrogen in the rumen towards acetic acid production by reductive dehydroxylation of phenolic compounds. Furthermore, when animals were treated only with chloroform (pre and post phloroglucinol) no significant differences on rumen fermentation parameters were observed within the groups.

Formate concentration increased as methane formation declined and hydrogen accumulated, in accordance with previous finding. However, when animals received phloroglucinol, formate concentration decreased as hydrogen expelled declined and methanogenesis remained inhibited. It is possible therefore, that formate might help in the control of metabolic hydrogen partial pressures in the rumen, playing a role as hydrogen sink and being an indicator of H_2 partial pressure as previously explained.

Our findings are in accordance with previous studies using phloroglucinol under *in vitro* conditions in ruminants, which observed an increased on acetic acid concentration (Tsai and Jones, 1975; Patel et al., 1981; Krumholz and Bryant, 1986; Murdiati et al., 1992) when phloroglucinol was degraded by rumen microorganisms. In accordance with our hypothesis, some authors (Krumholz and Bryant, 1986, Patel et al., 1981) have observed that rumen bacteria (*Eubacterium oxidoreducens* and *Coprococcus*) required H_2 or formate to degrade

phloroglucinol. Tsai et al. (1976) found that under anaerobic conditions *Coprococcus* degraded 1 molecule of phloroglucinol to 2 molecules of acetic acid and 2 molecules of carbon dioxide. The rumen microbiome study revealed OTUs assigned to *Coprococcus* genus positively affected by phloroglucinol + chloroform treatment compared with the animals only treated with chloroform. Furthermore, the increase in daily weight gain in the animals treated with phloroglucinol might be due to a hydrogen redirection towards alternative energy yielding pathways, such as the acetic acid production by reductive dehydroxylation of the phenolic compound.

On the other hand, no significant effects were observed on animals treated with the acetogenic bacteria when methanogenesis was inhibited (around 35% reduction), although some authors (Ungerfeld and Kohn, 2006) hypothesized that with a decrease in methanogenesis and subsequent increase in ruminal H_2 concentrations, reductive acetogens were able to compete successfully with methanogens for H_2 within the rumen. The unsuccessful results observed with this treatment compared with the *in vitro* results (Gagen et al., 2014) might be due to a possible sensitivity of the acetogenic bacteria to the chloroform or a higher dilution rate present in the rumen which could affect colonisation of that ecosystem by the bacteria. Chloroform is a halogenated, such as BCM, with a similar mode of action. BCM reacts with reduced vitamin B_{12}, inhibiting the cobamide-dependent methyltransferase step of methanogenesis (Wood et al., 1968; Chalupa, 1977). Acetogenic bacteria carbon metabolism also use the B_{12} dependent methyltransferases (Banerjee and Ragsdale, 2003), and therefore BCM and chloroform might affect reductive acetogenesis, which has not been investigated.

The study by quantitative PCR of the FTFHS gene, a key functional gene from the reductive acetogenesis pathway, revealed no significant effects between animals treated with the acetogenic bacteria or phloroglucinol compared with the positive control group (chloroform treatment). These results suggest that reductive acetogenesis pathways provide minor contributions to the redirection of H_2 with the additives/microbial treatments tested. Furthermore, existing tools targeting the FTHFS sequence as a marker for reductive acetogenesis are compromised by lack of specificity due to the involvement of this key gene in other pathways (Drake et al., 2008, Pierce et al., 2008). Future studies should test whether acetogens are able to compete successfully with methanogens under a complete methane suppression using dietary additives promoting them or inoculating acetogens resistant to the antimethanogenic compounds.

The third *in vivo* experiment to study the availability of dietary supplements to consume hydrogen confirmed the results observed from the second trial. We observed a shift towards more acetic acid production and a greater concentration of total SCFA in the rumen of animals fed with a lower fermentable diet (Rhode grass hay) and treated with phloroglucinol. However, expelled hydrogen was not detected with the medium dose of chloroform. The lack of effect on expelled hydrogen in chloroform groups might suggest a redirection of hydrogen which could be due to the high CP presented in the forage and the slower fermentation rate of the hay diet compare with the highly fermentable concentrate diet used in previous trials. In line with our findings, the first trial showed that greater amounts of H_2 (1.7 – 2.9 fold; g/kg DMI) were expelled in animals supplemented with the hay:concentrate diet compared to the hay only diet. Furthermore, an increase in daily

weight gain was also observed in the animals treated with phloroglucinol in accordance with the second trial which might be linked with the hydrogen redirection and a productivity improve. Regarding the rumen microbial community, OTUs assigned to *Coprococcus* genus were positively increased by phloroglucinol + chloroform treatment as showed in trial 2, which confirm *Coprococcus* as an important player for the degradation of phloroglucinol and redirection of H_2 towards acetic acid in the rumen.

The fourth *in vivo* experiment studied the effects of nitrogen supplements (urea and casein) on cattle fed with two different fermentable diets, slowly and highly degradable (hay and hay:concentrate respectively), when methanogenesis was inhibited. Nitrogen supplements can stimulate the bacteria and enhance fibre digestion in the rumen (Soto et al., 1994, Van Soest, 1994). Furthermore, the rumen microbial ecosystem might respond differently depending of the diet and the source of nitrogen used as supplement. The last trial examined the effect of different nitrogen sources on hydrogen and rumen fermentation when methane is inhibited, which might vary depending of the diet characteristics. The dose of choloroform-CD used, decreased methane production by 90% and 50% with the hay and hay:concentrate diet, respectively. The greater methane reduction observed could be due to a better fixation of the chloroform into the cyclodextrin matrix which might produce a slower release and a longer life of the compound in the rumen. Dry matter intakes and rumen fermentation parameters were negatively impacted by the high methane inhibition in most of the animals, with the exception of those animals treated with casein and urea and fed with hay or hay:concentrate, respectively. Ammonia concentration and DM degradation in the rumen was not negatively impacted in those treatments compared with the control animals (without dietary additives), which suggest that the rumen function and fibre degradation was not impaired for these treatments when methanogenesis was inhibited. Furthermore a significant reduction of hydrogen expelled per kg of DMI was observed in the urea treated animals fed with the highly degradable diet, which might suggest a hydrogen redirection to alternative energy pathways. Regarding the daily weight gain, no significant differences were observed between treatments although a much greater weight gain was reported from those groups treated with casein and urea and fed with hay or hay:concentrate, respectively. However, due to the negative impact that the methane inhibition had in 3 of the animals (one per group), the results from this last trial have to be taken carefully. The low number of animals per group (3 steers) at the end of the experiment did not allow to have enough statistical power to obtain significant differences for some of the parameters, although a pattern was observed. In the future, further experiments should be designed to confirm the patterns and results observed with the nitrogen dietary supplements.

The greater amounts of H_2 expelled in animals fed with the hay:concentrate diet compared to the hay only diet are in agreement with the results reported from all the trials. The rumen microbiota in the roughage hay fed animals seemed to utilise more H_2 that was available from the reduction in CH_4 formation than the hay:concentrate fed cattle, possibly due to the slower fermentation rate of the hay diet compare with the highly fermentable concentrate diet. This finding might be taken into consideration for designing future strategies to manage the hydrogen excess in the rumen.

5. Conclusions

In conclusion, the present project showed in cattle that a reduction in methane formation by 30-35% resulted in a redirection of H_2 into more reduced microbial end-products and eructation of excess hydrogen without an apparent adverse effect on DM intake, fibrolytic activity and general rumen function. The amount of expelled H_2 per mol of decreased methane was lower for the roughage hay diet suggesting a more efficient redirection of hydrogen perhaps due to the slower fermentation rate and evolution of H_2 compared with the hay:concentrate supplemented animals. The metabolomics analysis indicated the nitrogen supply may have been enhanced by increased proteolysis and microbial protein synthesis particularly in the methane inhibited cattle fed roughage hay. These changes in metabolism were accompanied by a shift in the microbiota to more Bacteroidetes and a decrease in archaea and Synergistetes for both diets. Synergistetes among other bacteria groups, may be sensitive indicators of H_2 partial pressures in the rumen. Although there was a redirection of H_2, dietary supplements or microbial treatments might be needed to drive the excess H_2 into energy-yielding substrates and consequently improve the energy supply to the animal. Our results suggest that the bacterial fibrolytic community was not affected by the increasing level of H_2, although the relative abundance of some particular OTUs changed. Further analyses to study how those changes affect the fibre degradability in the rumen should be carried out in future experiments. Supplementation of phenolic compound in animals demonstrated a redirection of hydrogen excess towards acetic acid production when methanogenesis was inhibited improving the energy supply to the animal. *Coprococcus* has been identified as the microorganism involved in that process. The significant increase in daily weight gain observed with some of the dietary treatments, which redirected hydrogen to energy yielding products, might improve the productivity by more than 10%. To achieve this increase in productivity, the rumen microorganism must have enough nutrients for enhance the microbial growth and metabolism. Future work should be focus on identify practical feeding farming strategies to capture this excess hydrogen as energy for the animal.

6. References

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7. Findings

1. Established a cattle model with methane inhibitor (chloroform) and hydrogen accumulation.
2. Prediction of H_2 flow under methanogenesis suppression developed in cattle.
3. Metabolomics analyses showed an increased in microbial protein and organic compounds under antimethanogenesis conditions.
4. Phenolic supplementation decreased H_2 and increased acetogenesis when methanogenesis was suppress in cattle.
5. Inoculation of acetogenic bacteria in the rumen of cattle did not show significant effects in animals treated with chloroform.
6. *Prevotella* and *Coprococcus* genus identified as possible hydrogen consumers under different rumen conditions.
7. A significant increase in daily weight gain observed with dietary treatments that redirected hydrogen when methane was inhibited.

8. Scientific publications

The preliminary results obtained from the first *in vivo* experiment have been presented as oral communication in the international conference: 'Joint ISNH/ISRP International Conference' the 9th of September 2014. Abstract title: "Rumen methane reduction mediated by chloroform increases expelled hydrogen in cattle."

Results from the second trial (phloroglucinol additive) presented as oral communication in the international conference: "6th Greenhouse Gas and Animal Agriculture Conference (GGAA2016)" 14th – 18th February 2016. Abstract title: "Phloroglucinol degradation in the rumen promotes the redirection of hydrogen when methanogenesis is suppressed"

Manuscript submitted to the international Journal "Frontiers in Microbiology' (26-04-2016). Manuscript title: "Methane inhibition alters the microbial community, hydrogen flow and fermentation response in the rumen of cattle".

Gagen, E.J., Wang J. K., Padmanabha, J., Liu, J., Pena Carvalho de Carvalho, I., Liu, J.X., Webb, R.I., Al Jassim, R.,Morrison, M., Denman, S.E. and McSweeney, C.S. (2014). Investigation of a new acetogen isolated from an enrichment of the tammar wallaby forestomach. BMC Microbiology 14:314 DOI: 10.1186/s12866-014-0314-3

Denman S. E., Martinez Fernandez G., Shinkai T., Mitsumori M. and McSweeney C.S. (2015) Metagenomic analysis of the rumen microbial community following inhibition of methane formation by a halogenated methane analog. Front. Microbiol. 6:1087. doi:10.3389/fmicb.2015.01087

9. Communication material

Article published in the MLA Feedback Magazine on rumen microbiology research progress.