Ministry for Primary Industries
Manatū Ahu Matua

Framework & Milestone report – H_2 management in the rumen

Final report

MPI Technical Paper No: 2018/30

SLMACC-AGR30783

Prepared for Ministry for Primary Industries
by Stefan Muetzel

ISBN No: 978-1-77665-884-8 (online)
ISSN No:

November 2015

New Zealand Government

Disclaimer

This report has been prepared for the Ministry for Primary Industries and is CONFIDENTIAL to that organisation and AgResearch. AgResearch will not disclose its contents to third parties unless direct to do so by Ministry for Primary Industries. Every effort has been made to ensure this publication is accurate. However, because research and development can involve extrapolation and interpretation of uncertain data, AgResearch will not be responsible for any error or omission in this publication unless specifically agreed otherwise in writing. To the extent permissible by law, neither AgResearch nor any person involved in this publication accepts any liability for any loss or damage whatsoever that may directly or indirectly result from any advice, opinion, representation, statement or omission, whether negligent or otherwise, contained in this publication.

Requests for further copies should be directed to:

Publications Logistics Officer
Ministry for Primary Industries
PO Box 2526
WELLINGTON 6140

Email: brand@mpi.govt.nz
Telephone: 0800 00 83 33
Facsimile: 04-894 0300

This publication is also available on the Ministry for Primary Industries website at
http://www.mpi.govt.nz/news-resources/publications.aspx

Contents

Executive summary

This programme contributed to SLMACC Theme 2, Agriculture: "methane from ruminant animals and soils". The project aims to determine if long-term hydrogen (H_2) accumulation in the rumen, when rumen methanogens are directly inhibited, will have negative effects on rumen function and consequently animal performance. This knowledge needs to be established before mitigation strategies such as vaccination and small molecule inhibitors can be applied on farm. These technologies, currently being developed in PGgRc-funded programs¸ are especially suitable for NZ pasture based systems and removing potential barriers to their adoption will be of major benefit for reducing CH_4 emissions.

The main objective of this project was to examine the effects of sustained methanogen inhibition and the accumulation of molecular H_2 in the rumen and subsequent effects on rumen function and animal production parameters. This would provide crucial knowledge to address potential barriers to adoption of CH_4 mitigation technologies. A second objective of this project was to evaluate if dosing animals with a methanogen inhibitor early in their life cycle, when the rumen microbial community may be less resilient to interventions, will affect CH_4 production in later in their adult life.

The key results in objective 1 were:

1) Increasing the concentration of molecular hydrogen through the inhibition of methanogens in the rumen of sheep did not negatively affect total tract fibre digestibility, a key parameter in ruminant nutrition that affects energy supply and therefore animal productivity.
2) Disposal of hydrogen produced during rumen fermentation in sheep and cattle appeared to be different. Cattle disposed a high proportion as molecular hydrogen while in sheep, molecular hydrogen was observed only during the adaptation phase of the sheep to the methanogen inhibitors.
3) Negative effects on voluntary feed intake in response to the inhibitors were stronger in sheep than in cattle. However, this effect appeared to be associated with negative reactions of the animals to the compounds, rather than the increase in the concentration of molecular hydrogen.

These results suggest that accumulation of molecular hydrogen in the rumen does not affect rumen function negatively, and therefore methane inhibition treatments like vaccines or small molecule inhibitors do not need to be supplemented with a hydrogen sink to remove the molecular hydrogen from the rumen. These results are valuable once methane mitigation technologies based on methanogen inhibition become commercially available because they demonstrate to end-users that methane inhibition can be achieved, in principle, without interfering with normal rumen function.

The key results from the objective 2 were:

1) Complete methane inhibition in calves did not affect their intake of milk and solid feed or growth performance.
2) The effects on rumen function and methane inhibition in early life did not lead to a metabolic imprint and methane emissions increased to similar levels as for control animals when the inhibitor treatments were stopped.
3) In contrast to observations with adult animals, the methane inhibitors fed to calves did not decrease their dry matter intake and the effects of methanogen inhibition on rumen metabolites were not as pronounced as observed in adult ruminants.

These results indicate that a short term early life intervention does not leave a metabolic imprint (i.e. decreased methane emissions) in the rumen and therefore does not present an inexpensive alternative to a long term inhibitor application or vaccination against methanogens. However, since the colonisation of the rumen with bacteria and therefore the susceptible time for intervention starts at birth, treatments based on solid feed may miss the most susceptible time to generate an microbial imprint since solid feed intake starts only at the second to third week of life. Inoculation of young animals at birth or within the first week could be a more promising strategy which may also have positive effects on the development of the hindgut flora and therefore animal health. The lack of an effect on methane emissions might also be related that the any possible imprint manifest itself only during conditions similar than those in early life. For the present experiments the testing conditions would be a high hydrogen rumen environment which is observed when concentrates rather than pasture is offered to the animals.

MS 1 Framework of final report

INTRODUCTION

There is still considerable uncertainty in the scientific community and the agricultural industry whether the accumulation of molecular hydrogen that occurs when rumen methanogens are inhibited, will have detrimental effects on animal performance. This key question has to be addressed before mitigation strategies based on inhibitors or vaccines are accepted by the farming industry. Inhibitor and vaccine based technologies are attractive for New Zealand (NZ) production systems because other alternatives, such as nutritional interventions, are limited in grazing systems. Previous attempts to inhibit methane emission using bromoethanesulfonate (PGGRC, Mitigas) and acetylene (SLMACC, C10C1105) have been unsuccessful because the treatment effects in sheep were only short lived. Methane analogues like bromochlormethane and chloroform have been used successfully to inhibit methanogenesis in goats, sheep and cattle. However these experiments were either very short-term (McCrabb et al., 1997) or focused only on microbial community analysis (Mitsumori et al., 2012). There is only one study where the relationship between the effect of a methane inhibitor and an animal production trait (milk solids in dairy goats) has been investigated (Abecia et al., 2012). Our aim is therefore to inhibit methanogenesis over a sufficient time-period to study the effect of these treatments on methane and hydrogen emissions as well as the fermentation end products, feed digestibility and production parameters in sheep and cattle.

A new and relatively unexplored strategy to inhibit methane is an early life intervention designed to either reduce hydrogen production or increase hydrogen tolerance in the rumen. Short-term interventions in the early life of an animal may provide a more cost-effective solution compared to continuous inhibitor delivery. Artificial-rearing of calves is commonplace in the NZ dairy (milk production) and dairy-beef (meat production) sectors. Therefore, this approach has the potential to be implemented with relative ease. The effects of early life nutrition on the development of the rumen (Berends et al., 2012) and general development of an animal is well documented (Soberon et al., 2012). However, the impact of early life nutrition on the microbial community in ruminants, methane emissions and subsequent animal performance in later life is poorly understood (Belanche et al., 2010, Abecia et al., 2012). We are aware of only one study where the effect of early life nutrition on the microbial community composition later on in life was studied (Yanez-Ruiz et al., 2008).

OBJECTIVES

1. Objective 1. "Methane free rumen": To demonstrate that a methane (CH_4)-free rumen continues to maintain normal functionality long-term.

   Impact: Understanding the implications of inhibiting rumen methanogens in livestock in NZ farming systems will impact the implementation of inhibitors or vaccines.

2. Objective 2. "Early life intervention". To determine whether short-term early-life intervention using inhibitors can lead to a permanently lower methane emission later in life.

   Impact: Novel approach to lower the cost of inhibitor dosing, and development of an approach that is compatible with NZ farming systems.

APPROACH

Objective 1: Methane free rumen

a) We will determine a suitable inhibitor combination to decrease methane emissions from sheep for a period of 4 weeks to study the effect of the treatment on hydrogen levels, rumen end products and microbial community composition.
b) The most promising treatment will then be used in to test the effect of treatments on live weight gain in lambs and milk production in dairy cattle.

Objective 2: Early life intervention

a) The long-term impact of short-term methane inhibition in early life will be evaluated in male calves by evaluating their methane emission profile and microbial community composition for 3 months following cessation of inhibitor treatment.
b) If successful, the treatments will be repeated with a larger group of female dairy calves to assess the impact of such a treatment on live weight gain, body condition score on milk production.

METHANE FREE RUMEN: INHIBITOR PILOT STUDY

The pilot study was a requirement of the Animal Ethics committee, to ensure that there are no immediate negative effects of 9,10-anthraquinone on sheep. The Animal Ethics Committee permitted some measurements to be undertaken on the animals in this study to provide preliminary data to support future experiments.

Design

A total of 9 animals were adapted to GP diet for 7 days and were then transferred into the respiration chambers. While in chambers dry matter intake (DMI) was determined on a daily basis. Methane and hydrogen was measured for one day without AQ added to the diet followed by three days of AQ added to the diet at 1, 0.5 and 0.1 g sheep/day.

Data were analysed by comparing each treatment group to the control period where no inhibitor was fed by one way ANOVA using Genstat v.15.

Diet

9,10-anthraquinone was purchased from Sigma (Cat.No.: A90004). The doses of AQ tested were 1.0, 0.5 and 0.1 g / per sheep and day leading to an effective concentration of 1, 0.5 and 0.1 mM assuming a 5 L rumen volume. AQ was ground to cellulose powder (1:2) to help dispersing in the rumen and mixed into a general purpose (GP) diet. This diet consists of 500 g chaffed hay, 290 g crushed barley, 100 g soy bean meal, 100 g molasses and 10 g salts and minerals.

Organic matter, lipid, crude protein sugar neutral and acid detergent fibre content was 944, 11, 133, 51, 438 and 221 g/kg DM respectively. Analysis was carried out by Massey University Nutrition Laboratory using the following methods for analysis: crude protein. Leco, total combustion method. AOAC 968.06; Lipids, Soxtec extraction. AOAC 991.36; ash, Furnace 550 °C, AOAC 942.05; Sugars, Phenol sulphuric ; NDF/ADF, (Robertson and Van Soest, 1981) . Tecator Fibretec System. AOAC 2002.04 (A.O.A.C, 1990).

Results & Discussion

Dry matter intake of the lambs was unaffected by AQ treatment and all but one animal ate the whole diet. The impact of AQ treatment on methane production was less than expected based on earlier *in vitro* studies. No difference in methane emissions in response to the inhibitor was detected for any of the different doses (Table 1) which is likely due to the small number of animals. Out of the three animals in the AQH treatment, two showed a decrease in methane emissions by 20% while methane emissions from the third animal increased. However, the hydrogen emission of this animal was increased as for the animals where methane emissions were inhibited indicating that the methanogens were inhibited in this animal as well. Hydrogen emissions on average increased significantly with increasing inhibitor concentration in the medium AQ treatment (Table 2). Across the treatment groups hydrogen increased dose dependent but was not significant in the high treatment group. There was a clear feeding pattern in the emissions of hydrogen as shown in Figure 1 where hydrogen levels increase in response to feeding at 0800 and 1600h.

No toxic or adverse effects of AQ were observed in the sheep during this study, and the animal ethics committee agreed that based on the data collected the trials with AQ can be undertaken. .

Table 1: Methane yield (g/kg DMI) for sheep fed three different levels (high 1.0g, medium 0.5 g and low 0.1g per sheep) of 9,10-anthraquinone.

Day	Treatment	AQ low	AQ medium	AQ high
1	GP	25.7	27.2	23.6
2	GP & AQ	27.2	26.3	19.4
3	GP & AQ	25.9	24.9	18.9
4	GP & AQ	26.3	24.6	20.1
	SED	0.84	0.98	2.75
	P value	0.356	0.078	0.351

SED, Standard error of the difference

Table 2: Hydrogen yield (g/kg DMI) for sheep fed three different levels of 9,10-anthraquinone.

Day	Treatment	AQ low	AQ medium	AQ high
1	GP	0.06	0.02	0.02
2	GP & AQ	0.08	0.13	0.32
3	GP & AQ	0.10	0.18	0.58
4	GP & AQ	0.08	0.17	0.53
	SED	0.030	0.015	0.200
	P value	0.627	<.001	0.060

SED, Standard error of the difference

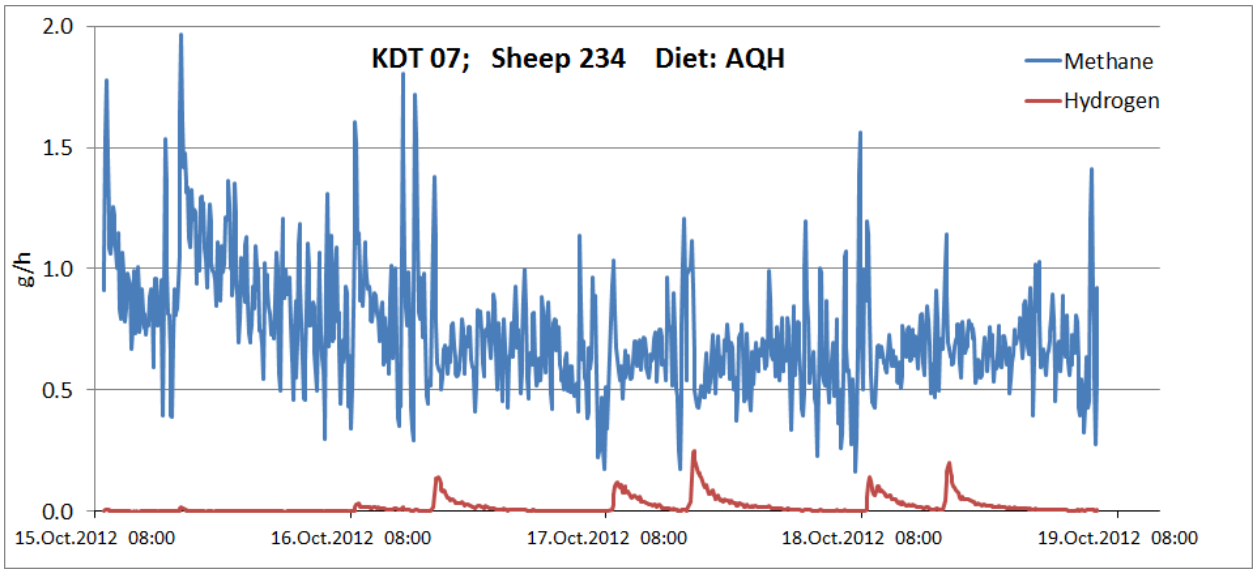

Figure 1. Daily pattern of methane and hydrogen emissions of a sheep fed anthraquinone at a dose of 1 g/d.

Although animal numbers were small and the effects observed in this short study were not large, the increased hydrogen emissions observed when AQ was fed indicated that AQ is a suitable inhibitor to study the effect of hydrogen on rumen function. In addition a study with sheep showed that the microbial community in the rumen does not adapt to AQ over a period of 3 weeks (Kung Jr et al., 2003), which is crucial when effects of methane inhibition on production parameters is examined. Based on this study the level of AQ used in further experiments will be 1 g/kg DM.

However in order to increase the magnitude of the hydrogen effect AQ will have to be used in combination with another inhibitor. Chloroform has also shown methane inhibition over a

period of several weeks (Knight et al., 2011) and is a more potent inhibitor, based on our in vitro studies.

Objective 1, MS 2 &4; Effects of methane inhibition on hydrogen disposal and fibre digestibility.

In ruminants the major route of hydrogen disposal is methane formation. Early experiments with showed that fibre degrading bacteria growth better when co-cultured with a methanogen that removes the hydrogen from the fermentation. And based on these results it was concluded that hydrogen accumulation in the rumen will lead to decreased fibre degradation with negative effects on animal productivity.
Using sheep we examined the effect of methane inhibition on molecular hydrogen production, short chain fatty acid composition, total tract dry matter and fibre degradation along with the response of the methanogen and bacterial community in the rumen. Chloroform and 9,10-anthraquinone, both potent methane inhibtitors, were added to a total mixed ration and offered to the sheep. The experiment consisted of three periods, a baseline, an inhibitor and a recovery period. Two treatments, a combination or a sequential application of the inhibitors were compared to a control group. Complete methane inhibition was only achieved during the first 10 to 14 days of the inhibitor period and significant amounts of molecular hydrogen (H_2) were observed only during the first week of inhibition. Only slightly higher H2 production was observed during the remaining treatment time. During methane inhibition, the production of acetate was decreased and that of propionate, another hydrogen disposal route, was increased. Besides propionate the main hydrogen disposal route in the trial was formic acid. In the sheep methane inhibition did not affect dry matter or fibre digestibility negatively, in fact a positive effect was observed for both treatment groups during the inhibitor period. The methanogen community showed a sequential response to the inhibition where the proportion Methanobrevibacter gottschalkii was decreased to be replaced by Thermoplasmatales initially followed by M. ruminantium. When the inhibitor treatment was stopped M. gottschalkii was the dominant species again. The bacterial community was differed between the treatment and the control group as well as between the combination and sequence treatment. Groups that were positively related to the treatments included Treponema, Succinivibrio and Prevotella. Within the fibre degrading community only a decrease in Ruminococci during the treatment period was observed.
We conclude that an accumulation of H2 in the rumen does not occur after an initial adaptation phase and that the major hydrogen removal routes are short chain fatty aids and therefore no negative effects on fibre degradation were observed.

INTRODUCTION

In the rumen the reduction of carbon dioxide to methane is the major route of hydrogen disposal. However methane is a greenhouse gas 25 times more potent than carbon dioxide and makes up to 45% of the greenhouse gas emissions in New Zealand (Clark et al., 2011). Methane is also a loss of gross energy to the ruminant that can account for 2 to 12% (Johnson and Johnson, 1995) and inhibition of methane emissions seems to be beneficial in both ways to the animals and the environment. However there is concern that disrupting this process can negatively influence rumen fermentation and consequently dry matter intake and animal productivity. When methane emission are inhibited one of the common products of rumen fermentation is molecular hydrogen (Janssen, 2010) which is postulated to be detrimental to some rumen microorganisms. Positive effects on cell growth or enzyme activity have been demonstrated for co-cultures of bacteria and fungi when the hydrogen in the culture was removed by a methanogen (Latham and Wolin, 1977, Teunissen et al., 1992, Williams et al., 1994). Many of these co-cultures have been carried out with important rumen fibre degrading species like Ruminococcus flavefaciens and rumen fungi and therefore it is assumed that hydrogen accumulation will negatively affect ruminal fibre degradation. It has been therefore suggested that methane inhibition should be accompanied by the application of hydrogen

sinks to decrease the negative effects of the hydrogen on fermentation (McAllister and Newbold, 2008). However, in pure cultures the hydrogen concentration, especially the dissolved hydrogen concentration can increase substantially, since the system is closed and pressurised. In contrast in a ruminant the hydrogen pressure is expected to be much lower because hydrogen in the headspace can escape into the bloodstream or with the ructus.
In this experiment we were trying to determine the effect of methane inhibition on ruminal hydrogen disposal and its effect on total tract digestibility of dietary fibre. In addition we examined the effects on the bacterial and methanogen community composition.

MATERIAL AND METHODS

A mob of 24 sheep (49.7 ± 4.04 kg live weight) was used in this experiment and randomly allocated to three groups of animals. One group served as control whereas the two other groups had methane inhibitors offered to them mixed into their diet. Two approaches for inhibition of methanogenesis were compared: 1) application of the inhibitors in sequence and 2) the combination of the two inhibitors. These two treatments will be compared to a control group of animals to determine which treatment works most effective over time. The inhibitors chosen were 9,10-anthraquinone (AQ) and chloroform (CF) based on their potential long term effects.
The experiment was carried out in three periods. During the baseline period all animals received only control diet to establish pre-treatment values for methane emissions, fermentation parameters and total tract digestibility. Afterwards the treatments were started and one grup received a sequence of inhibitors while the second group received the combination of the inhibitors and the third group served as a control group. This was followed by a recovery period during which all animals received only the control diet with no inhibitors added.

Diets

The control diet (general purpose diet, GP) contained 500 g Hay, 290 g Barley, 100 g Soya, 100 g, Molasses, 5.5 g Di-calcium-phosphate, 3.0 g Salt and 1.5 g Mineral-mix. The diet contained 78 g/kg ash, 148 g/kg crude protein, 15 g/kg lipids, 431 g/kg NDF and 327 g/kg sugars. The methane inhibitors were added into the diet. Earlier work in vitro indicated that particle size of the AQ increases its effectiveness against methanogens and AQ was therefore ground onto cellulose before mixing into the diet. The second inhibitor, CF was complexed with β-cyclodextrine to yield a white solid that contained 100 g/kg CF and could be mixed into the diet. The concentrations AQ and CF in the diet were 1.0 and 0.125 g/kg. Both inhibitors were then premixed into the soybean meal using a commercial food blender before mixed into the final GP diet in a concrete mixer. Diet containing the inhibitors was made up fresh every 3 days and was stored in a chiller at 4°C until offered to the animals.
A total of 1.0 kg DM of the diet was offered in to equal meals at 0800 and 1600h to the animals corresponding to a feeding level of 1.5 x the maintenance energy requirement throughout the experiment. The diet was analysed form crude nutrient composition by the Nutrition Lab at Massey University, Palmerston North. Neutral detergent fibre (NDF), acid detergent fibre and lignin (ADL) were determined using the Tecator Fibretec System (Leco Corporation, St. Joseph, MI, USA) following the procedures of Robertson and Van Soest (1981). Lipid was determined by Soxtec extraction (Soxtec System AT1043 Extraction unit, Foss, Höganäs, Sweden) using method 920.39 of AOAC (2000). Total nitrogen (N) was determined in a Leco analyzer (AC350, Leco Corporation, St. Joseph, MI, USA) by the total combustion method (Method 968.06 AOAC, 2000), with crude protein (CP) calculated as total N multiplied by 6.25. Ash was measured by combustion in a furnace (Ceramic Engineering, Sydney, Australia) at 500°C for 16 hours (Method 942.05, AOAC, 1990) and

starch determined by the α-amylase method (Method AOAC 996.11, 2003). DMI was determined throughout the control and the treatment period where animals were kept either in single pens or in metabolic crates.

Sampling and analysis

Refusals were collected daily in the morning before new feed was allocated. During the recovery period the animals were group fed in pens most of the time and DMI was determined only during a 3 day adaptation period and measurement periods for methane or digestibility. Feed refusals were dried in a ventilated oven at 65°C for 48h and dry matter (DM) of the samples was determined gravimetrically by weight difference.

A timeline for the measurements and samples taken is given in Table 1. Methane measurements were carried out in respiration chambers for 48 hours except for the start of the inhibitor treatments where the animals where in chambers for 96 hours to assess the immediate effect to the inhibitors on methane and hydrogen concentrations. During the inhibitor period frequent methane checks were performed where the animals where kept for 2 – 3 hours in chambers to assess the effectiveness of the inhibitors. Design and use of the respiration chambers are described in (Pinares-Patiño et al., 2008).

At the end the control, treatment and recovery period faecal samples were collected over a period of 6 days to assess total tract apparent dry matter and fibre digestibility. Faecal samples were collected over 5 days in faecal bags in the morning. Samples were weighted pooled for each animal and stored at -20°C until analysis. Two representative aliquots of the faecal samples were dried in a ventilated oven at 65°C for 48h and dry matter (DM) of the samples was determined gravimetrically by weight difference. The dried samples were then ground in a hammer mill to pass a 1 mm sieve and were sent for analysis of DM, ash, NDF and ADF content to the Nutrition Lab of Massey University Palmerston North.

Rumen fluid samples were collected via stomach tubing at 4 occasions during the control period, 5 samples were collected during the treatment period and 3 during the recovery period (Table 1). Subsamples for DNA extraction (0.9 ml), analysis of short chain fatty acid (SCFA) and ammonia (NH_4) concentration (1.8 ml) were collected from the rumen samples. DNA samples were stored at -20°C until analysis, while SCFA acid samples were centrifuged at 21,000g at 4°C for 10 min. 0.9 ml of the supernatant were added to 0.1 ml of internal standard (19.8 mM ethylbutyric acid in 20 % v/v ortho-phosphoric acid) and frozen for at least 16 h. Samples were then centrifuged again and 0.1 ml of the supernatant was collected for ammonia determination by a downscaled method of (Weatherburn, 1967). The remaining supernatant was transferred into a 2 ml glass vial for SCFA analysis by gas chromatography. SCFA were determined in a Hewlett-Packard (HP) 6890 series GC with an auto-injector and flame ionization detector. The column was a Zebron ZB FFAP, the detector temperature was held at 240°C and the oven temperature was increased from 85 to 180°C at 10°C/min and held for 10 min. The carrier gas was helium at 5.5 ml/min. SCFA were quantified from a standard curve of known concentrations of acetate, propionate, butyrate, valerate, caproate, isobutyrate and isovalerate.

Table 1 Timeline of events for rumen sampling (RF) Methane measurements (CH_4) short term methane checks (CH_4 c) and digestibility measurements (Dig)

Period	Date		Samples
Control	30/10/2012		RF
	31/10/2012 to	1/11/2012	CH_4
	2/11/2012		RF
	9/11/2012		RF
	12/11/2012 to	17/11/2012	Dig
	18/11/2012		CH_4
Treatment	19/11/2012 to	22/11/2012	CH_4
	19/11/2012		RF
	23/11/2012		RF
	26/11/2012		CH_4 c
	29/11/2012		CH_4 c
	30/11/2012		RF
	30/11/2012		CH_4 c
	4/12/2012 to	5/12/2012	CH4
	6/12/2012		RF
	10/12/2012		CH_4 c
	10/12/2012		RF
	12/12/2012 to	17/12/2012	Dig
	18/12/2012 to	19/12/2012	CH_4
	20/12/2012		RF
Recovery	24/01/2013		RF
	28/01/2013		RF
	29/01/2013 to	30/01/2013	CH_4
	4/03/2013 to	9/03/2013	Dig
	11/03/2013		RF
	12/03/2013 to	13/03/2013	CH_4

DNA extraction was carried out using a phenol-chloroform protocol combined with mechanical cell disruption (Rius et al., 2012). This method was modified slightly for DNA extraction from liquid samples as follows. A subsample of 200 µl of whole rumen fluid was added to 200 µl 20% SDS, 182 µl buffer A (200 mM NaCl, 200 mM Tris, 20 mM EDTA, pH 8), 168 µl buffer PM (QIAquick 96 PCR purification kit, Qiagen, Valencia, CA, USA), 550 µl phenol-chloroform-isoamyl alcohol mixture (25:24:1, pH 8) and 0.7 g 0.1 mm zirconium beads. A BioSpec mini-beadbeater-96 (BioSpec Products Inc., Bartlesville, OK, USA) was employed for cell disruption at 2100 oscillations/min for 4 min. After centrifugation (20 min, 14 000 rpm, 4˚C), 350ul of supernatant was mixed with 650 µl buffer PM (Qiagen) before transferring and binding DNA to the membrane of a Qiagen QIAquick 96-well plate. After washing bound DNA with buffer PE (Qiagen), elution was performed by the addition of 100 µl elution buffer (Qiagen) under vacuum. Eluted DNA was quantified using an Invitrogen Quant-iT dsDNA assay, broad range kit (Life Technologies, Carlsbad, CA, USA) and FlexStation 3 microplate reader fluorimeter (Molecular Devices, Sunnyvale, CA, USA). PCR amplification of bacterial and archaeal 16S rRNA genes, and ciliate protozoal 18S rRNA genes was carried out following a method by (Kittelmann et al., 2013). The primers used were synthesized by Integrated DNA Technologies Inc. (Coralville, IA, USA) and consisted of a 454 Life Science adaptor A (5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG-3') or

B (5'-CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG-3') for titanium sequencing, a 2-base linker, a template specific sequence, and a unique Golay barcode attached to adaptor A (for unidirectional sequencing) for each sample, to allow for sample identification after amplicon pooling. Triplicate PCR products from each microbial group for each sample were pooled and product size checked by agarose gel electrophoresis. Following the quantification of the pooled triplicates (using an Invitrogen Quant-iT dsDNA assay, broad range kit (Life Technologies) and FlexStation 3 microplate reader (Molecular Devices)), pooling of PCR products for each microbial group into separate vials was performed. Products were pooled in varying volumes to achieve 150 ng PCR product from each sample in the three vials: bacteria, fungi, and protozoa. The pooled vials were then quantified using Invitrogen Qubit dsDNA assay, high sensitivity kit (Life Technologies) and an Invitrogen Qubit fluorimeter (Life Technologies). 1 µg of DNA from each pooled vial was loaded onto an agarose gel (1% wt/vol) and each band was excised under an Invitrogen Safe Imager 2.0 Blue-Light Transilluminator (Life Technologies). Each band was purified using a QIAquick gel extraction kit (Qiagen) and then quantified in triplicate using a Qubit dsDNA assay, high sensitivity kit (Invitrogen). The purified pools were diluted to $1x10^9$ molecules/µl and mixed at the ratio 5:1:1 bacteria:archea:protozoa PCR products. The products were then sent for 454 sequencing to MWG, Braunschweig, Germany.

RESULTS

Virtually no feed refusals were detected during the baseline and the recovery period and no differences in DMI were observed while the animals were in respiration chambers (Table 2). The differences in DMI for the control animals across the three periods are due to slight differences in the DM content of the diet offered. However DMI was decreased in treated animals during the inhibitor period. In fact after 4 days of treatment three of the animals in the combination group and two in the sequence group were removed from the experiment because their DMI was decreased by more than 50% for more than a day. The effect on DMI was very animal specific and some animals did not show any decrease in DMI in response to the inhibitor treatment. Apart from overall DMI, the inhibitors decreased the time in which the diet was consumed. In the control animals the diet was consumed within an hour after feeding while the animals receiving the inhibitors nibbled at their diets more or less constantly.

Table 2 DMI, methane and hydrogen emissions from sheep where methane emissions were inhibited by either a combination or a sequence of anthraquinone and chloroform.

		Treatment			
Period	Control	Combination	Sequence	sed	P value
	DMI [kg/d]				
Baseline	1.01	1.01	1.01	0.000	1.000
Inhibitor	1.01	0.91	0.95	0.038	0.050
Recovery	0.99	0.99	0.99	0.000	1.000
sed	0.000	0.043	0.020		
Pvalue	<0.001	0.076	0.016		
	CH_4 [g/kg]				
Baseline	25.7	26.4	26.6	0.92	0.552
Inhibitor	27.1	8.2	10.6	1.40	<0.001
Recovery	29.9	28.5	28.6	1.04	0.312
sed	0.64	1.38	1.49		
Pvalue	<0.001	<0.001	<0.001		
	H_2 [g/kg]				
Baseline	0.01	0.00	0.00	0.004	0.023
Inhibitor	0.10	3.75	3.69	0.331	<0.001
Recovery	0.01	0.04	0.02	0.017	0.104
sed	0.028	0.266	0.268		
Pvalue	0.003	<0.001	<0.001		

Methane yield (yM, g/kg DMI) did not differ between the treatment groups during the baseline period or the recovery period (Table 2). Only during the inhibitor period yM was lower in treated animals. The combination and sequence treatment decreased yM to a similar extent by 74 and 68% respectively. Across the trial periods yM was lower during inhibition in the treatment groups and no difference between baseline and recovery period was observed. However, an increase in yM was observed for the control group animals across the three measurement periods.

Hydrogen yield (yH, g/kg DMI) during the baseline period was higher in the control group but at a very low level (Table 2). During the treatment period the yH was increased in the treated animals compared to the control group and during the recovery period yH was similar between all groups. Across the periods an increased yH in the control animals was also observed, which might be related to a small carryover in the analyser due to the slower response of the hydrogen sensor compared to the other gases analysed. It is interesting to note that hydrogen emissions were observed only in the first few days of the inhibitor period and a week after the treatment started only minor hydrogen emissions were detected (Figure 1) although methane emissions were still very low. Over time methane emissions increased in the sheep but remained significantly lower until the end of the treatment period.

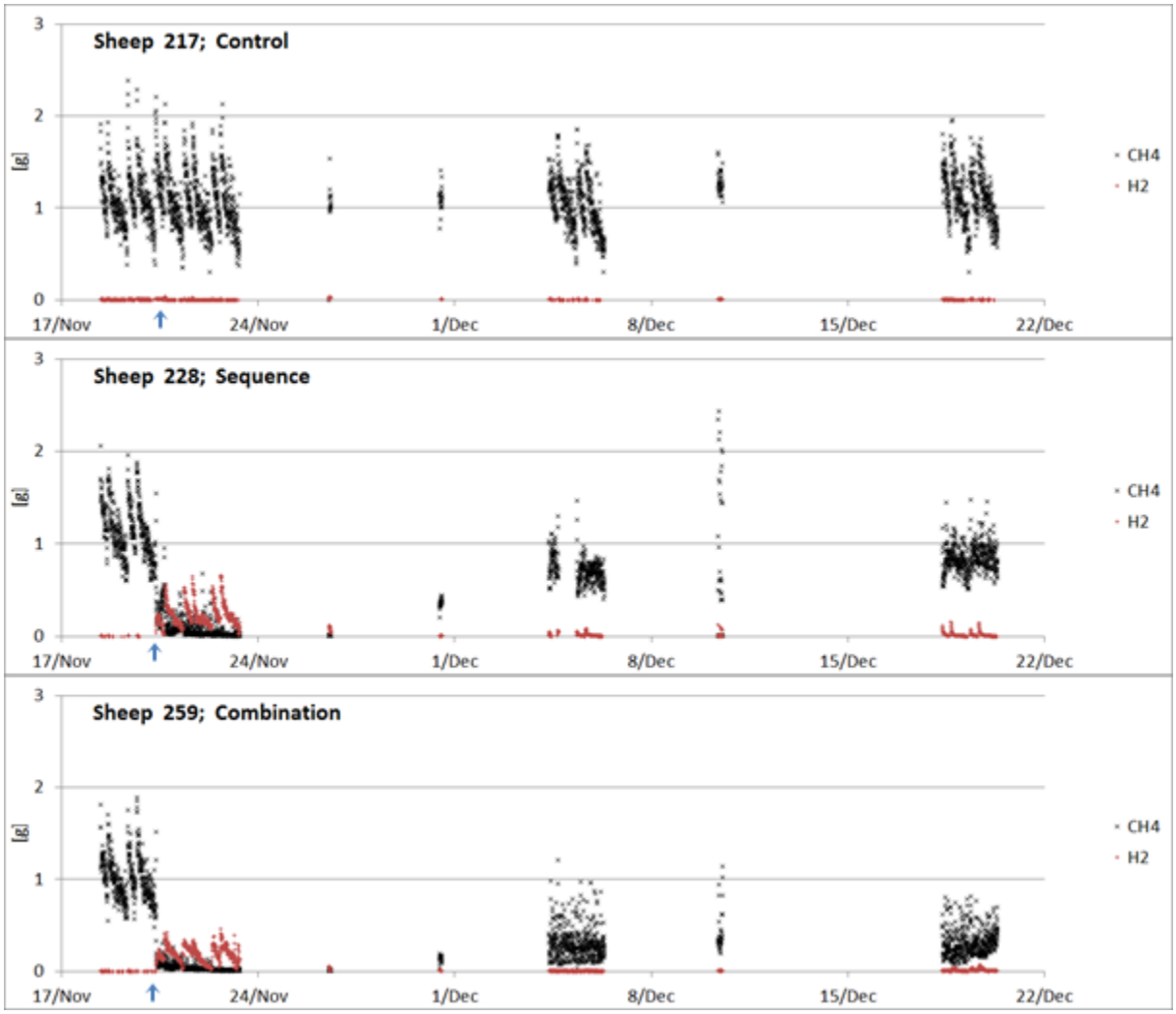

Figure 1 Methane and hydrogen emissions of the last two days of the baseline period and the inhibitor period of three selected animals of the three treatment groups measured. The arrows indicate the start of the treatment period where inhibitors were added to the diet.

During the baseline and the recovery period no difference in DM, NDF or ADF digestibility between the treatment groups was observed (Table 3). During the treatment period no difference in DM digestibility (DMD) was observed between the control and any of the treatments. NDF and ADF digestibility however were increased in both treatments compared to the control. However we also observed as decrease in DMD ADF and NDF digestibility over time in the control group and both the treatment groups.
Between the treatment groups the ruminal SCFA concentration did not differ during the baseline and the recovery period (Table 4). During the inhibitor period, the SCFA concentration in the animals receiving the inhibitors was lower compared to the control animals. Across all periods no differences in SCFA concentrations were observed for the control and the sequence group but the SCFA concentration of the combination group was lower during the inhibitor period.

Table 3 Effect of AQ and CF on apparent DM, NDF and ADF digestibility.

		Treatment			
Period	Control	Combination	Sequence	SED	P value
DMD [g/kg]					
Baseline	670	664	669	15.6	0.919
Inhibitor	656	638	674	11.5	0.031

Recovery	616	616	605	9.6	0.454
SED	12.9	10.6	11.2		
P value	0.001	0.002	<0.001		
NDFD [g/kg]					
Baseline	543	537	544	26.0	0.969
Inhibitor	429	467	539	21.0	<0.001
Recovery	396	400	390	17.0	0.856
SED	22.4	20.3	18.0		
P value	<0.001	<0.001	<0.001		
ADFD [g/kg]					
Baseline	462	449	460	32.4	0.912
Inhibitor	293	402	505	25.2	<0.001
Recovery	218	226	223	20.9	0.925
SED	27.7	24.3	22.4		
P value	<0.001	<0.001	<0.001		

SED, standard error of the differnce

The proportion of acetate was decreased in the combination and sequence treatment during the inhibitor period and the proportion of propionate was increased. No differences between the treatment groups were observed during the recovery period for either of the acids, but during the baseline period the proportion of acetate in the control animals was lower and the proportion of propionate was higher. Across the periods the proportion of acetate increased in the control animals while the proportion propionate was decreased. The acetate to propionate ratio (A/P) in all animals increased during the baseline period in all treatment groups and plateaued for the control animals at a value of 4 (

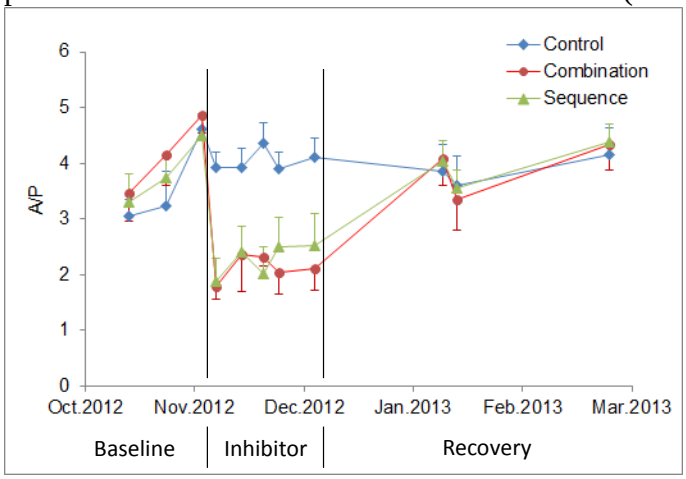

Figure 2). In the treated animals the ratio decreased to around 2 and increased to 4 again when the inhibitors were removed from the diets. No difference in the proportion of butyrate between the treatment groups was observed in any of the periods but butyrate proportion was increased in the control animals during the inhibitor period.

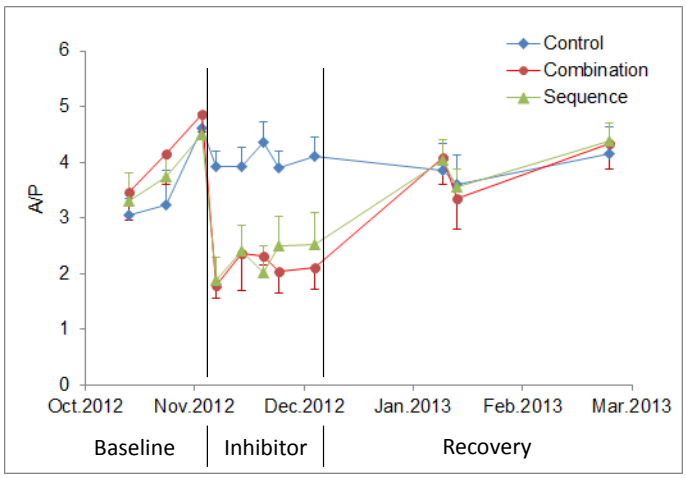

Figure 2 Acetate propionate ratio of treated (combination and sequence) and control sheep during the three experimental periods.

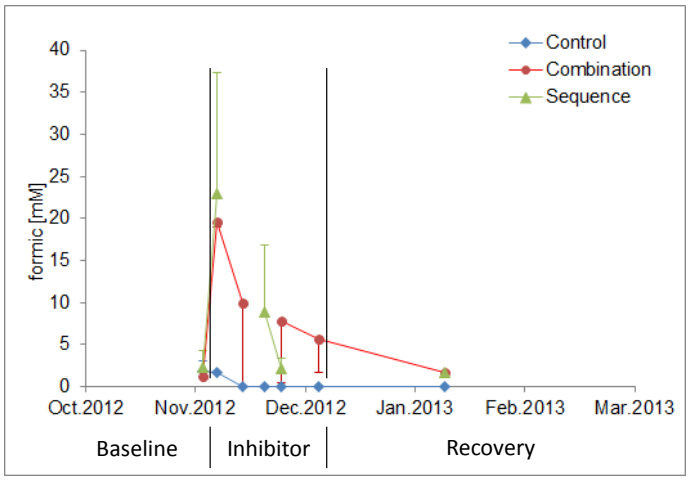

Figure 3 Formic acid concentration of treated (combination and sequence) and control sheep during the three experimental periods.

Table 4 Rumen fermentation parameters when methane emissions are inhibited by AQ and CF

		Treatment			
Period	Control	Combination	Sequence	sed	P value
SCFA [mM]					
Baseline	75.0	79.1	73.8	2.64	0.173
Inhibitor	78.7	66.5	71.4	3.14	0.004
Recovery	78.5	79.8	76.0	4.59	0.811
SED	2.89	2.82	4.53		
P value	0.381	<0.001	0.583		
Acetate [%]					
Baseline	62.9	65.0	64.0	0.62	0.011
Inhibitor	63.8	52.8	53.5	0.70	<0.001
Recovery	65.0	64.8	65.0	0.73	0.963
SED	0.48	0.88	0.76		
P value	0.001	<0.001	<0.001		
Propionate [%]					

Baseline	18.2	15.7	16.5	0.87	0.025
Inhibitor	15.8	25.8	24.9	1.36	<0.001
Recovery	17.0	16.8	16.6	0.75	0.754
SED	0.63	1.33	1.24		
P value	0.005	<0.001	<0.001		
Butyrate [%]					
Baseline	14.5	14.9	15.0	0.82	0.793
Inhibitor	15.7	16.4	16.5	0.86	0.587
Recovery	14.5	15.0	14.8	0.41	0.292
SED	0.46	1.09	0.74		
P value	0.021	0.386	0.095		
Minor [%]					
Baseline	3.0	3.1	3.1	0.21	0.896
Inhibitor	3.4	3.4	3.6	0.26	0.663
Recovery	2.7	2.6	2.6	0.16	0.765
SED	0.15	0.33	0.19		
P value	<0.001	0.115	<0.001		

In the control animals the methanogen community was dominated by Methanobrevibacter gottschalkii which made up between 66 and 86% of the total methanogen community (Figure 4A). Other major groups are Methanobrevibacter ruminantium, Methanosphaera sp. and Thermoplasmatales which contributed on average 9, 2 and 13% respectively. During the baseline and the recovery period the methanogen composition of both treatment groups was similar to the control animal. During the inhibitor treatments the methanogen community in the combination and sequence group showed a succession where the community was dominated by the Thermoplasmatales for one week after inhibition followed by M. ruminantium which became the dominant species for the remaining inhibitor period (Figure 4B and C). After inhibitors were removed from the diet, the methanogen composition of both treatment groups was not distinguishable from the control animals.

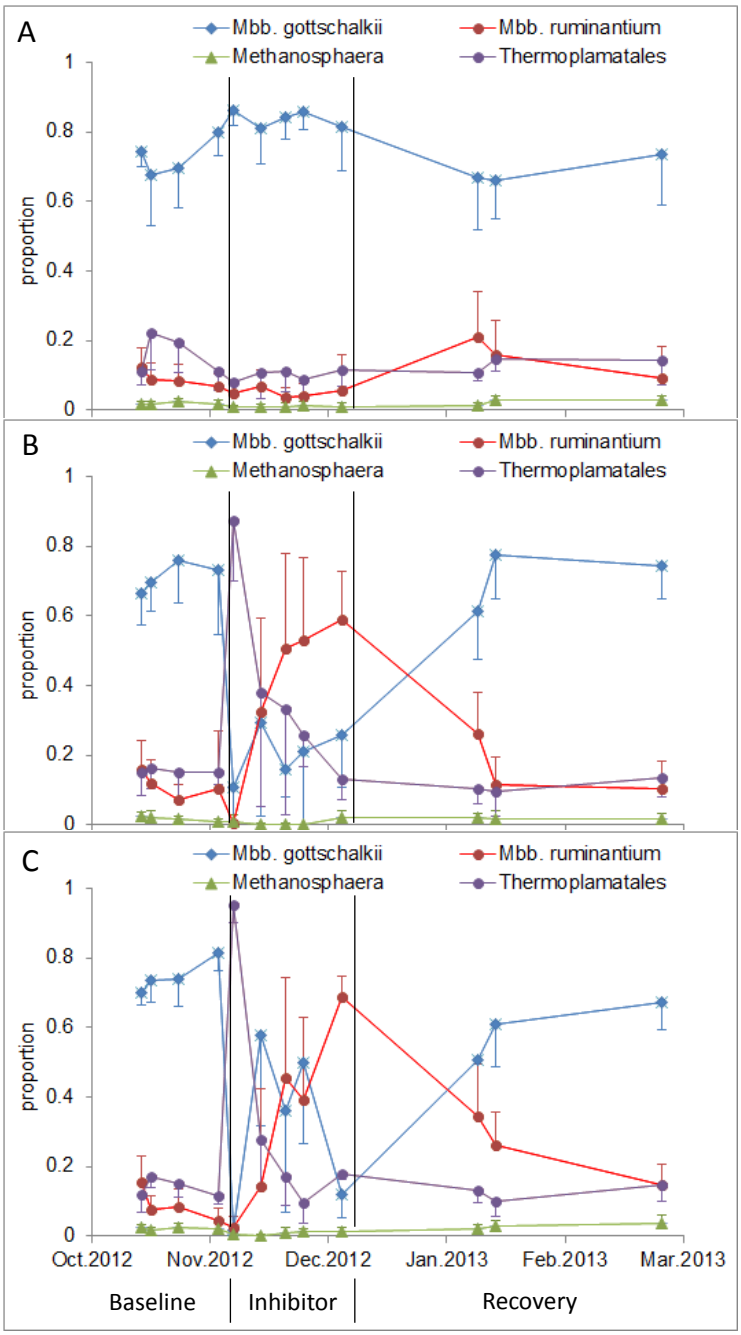

Figure 4 Methanogen community composition (C and S) and untreated (GP) sheep during the

Changes in the bacterial community were analysed by multidimensional scaling method and the species associated with changes were determined by correspondence analysis. During the baseline period the treatment groups were statistically separable ($P < 0.01$), the species associated with this separation were Palaudibacter, an unknown genus in the family of Prevotellacae and an unknown genus in the family of the Veillonellacae.
During the inhibitor period, the treatment groups separated from the control group ($P < 0.001$) as shown in Figure 5 and a canonical distance analysis revealed also a separation of the two treatment groups although to a much lesser degree (Figure 6). The sequence group was mostly influenced by increases in the proportion of Palaudibacter and an unknown genus in the family Veillonellaceae, while the combination group showed a shift towards Oscillospira and an unknown genus in the family Succinivibrionaceae.

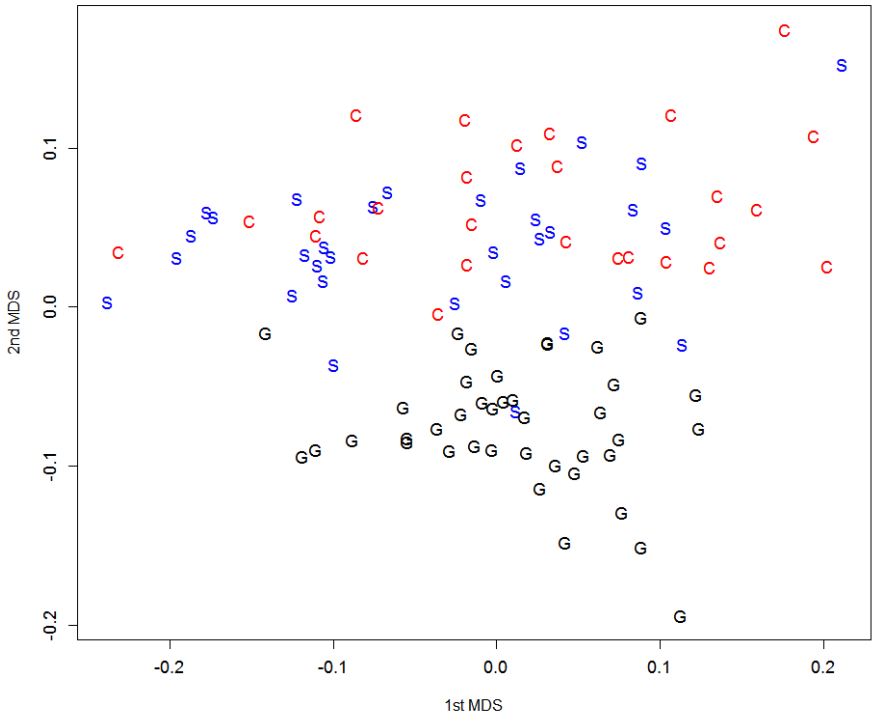

Figure 5 Separation of the bacterial community into treatment groups by multidimensional scaling during the inhibitor period.

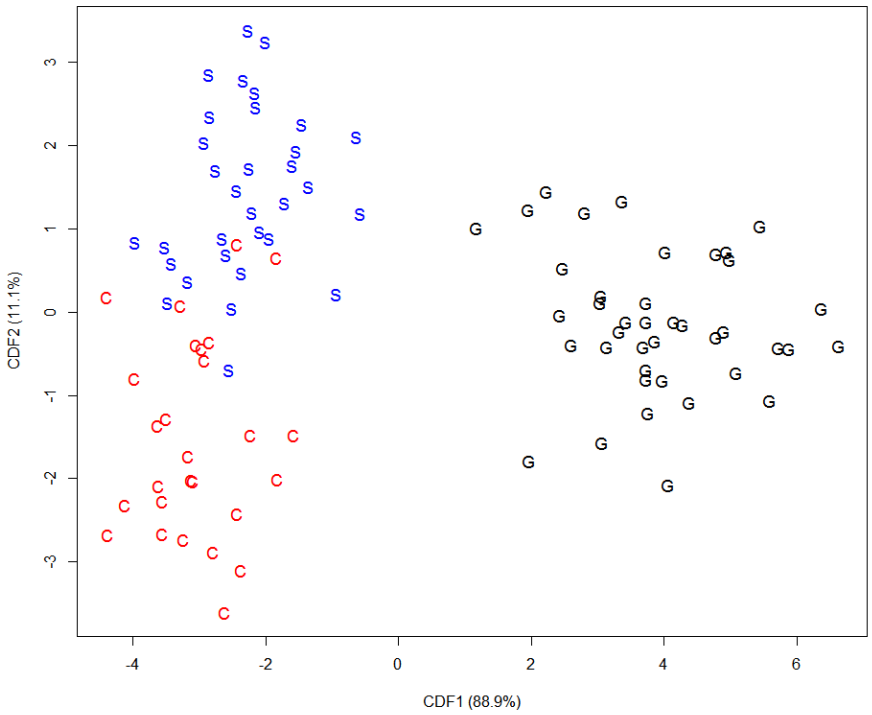

Figure 6 Canonical discriminant analysis of the bacterial community during the inhibitor period

Fibre degrading bacteria currently known and in culture are shown in Figure 7. Although no significant differences between any of the three groups were observed there was a general trend of lower Ruminococci during the treatment period but no difference between baseline and the recovery period was observed. Butyriovibrio and Fibrobacter did not seem to be affected by treatments.

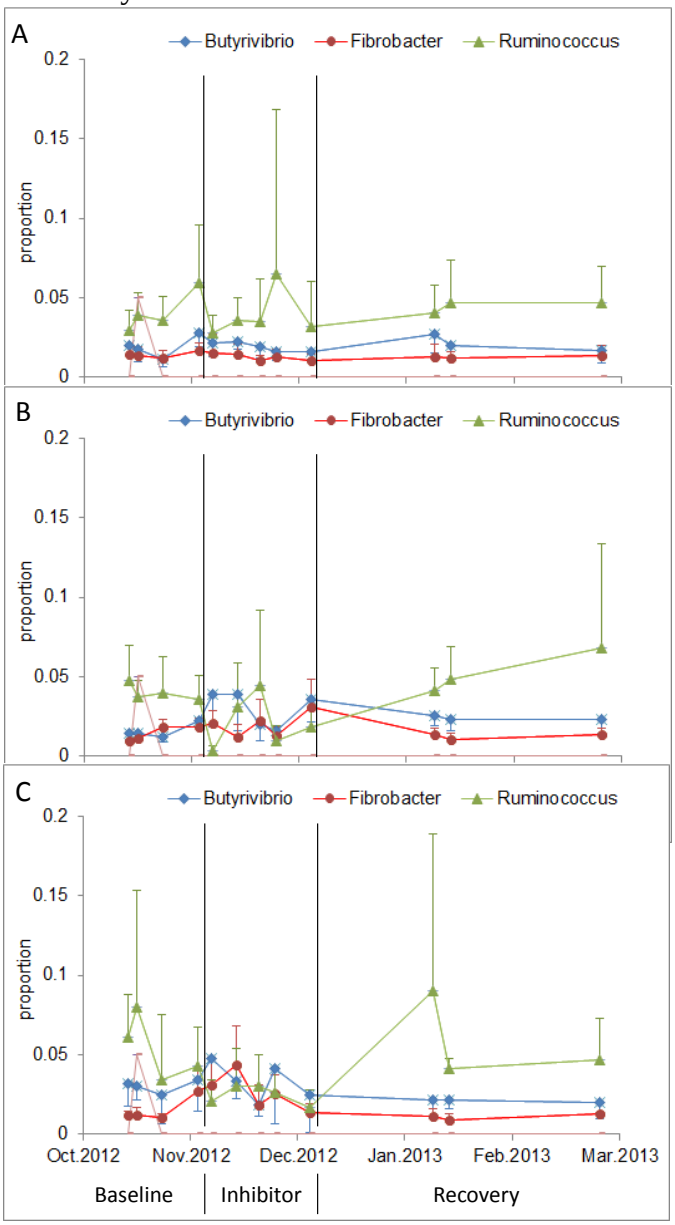

Figure 7 Distribution of the bacterial fibre degrading community for the control group (A), sequence group (B) and the combination group (C).

After the inhibitor period, no separation of the treatment groups was observed when analysed by multidimensional scaling (data not shown). However a distance analysis (
) showed that the distance between the communities of the inhibitor and the recovery period is less than the distance between the inhibitor and the baseline period. The distance between the baseline and the recovery period was higher in the treated groups compared to the control group.

Table 5 Pairwise squared Mahalanobis Distance between the periods for the three groups

	Recovery period	Baseline period
Control group		
Baseline period	15.0	
Treatment period	19.3	13.0
Sequence group		
Baseline period	26.3	
Treatment period	83.5	120.0
Combination group		
Baseline period	36.6	
Treatment period	51.5	89.1

DISCUSSION

Reduction of CO_2 to methane is the principal route of hydrogen disposal in the rumen, and compared to other hydrogen disposal routes like sulphate or nitrate reduction the end product is inert and the precursor is CO_2 which is abundant in the rumen environment. Hydrogen in the rumen is recognised to have a negative effect on rumen fermentation. Co-cultures of rumen bacteria and fungi with and without methanogens have induced differences in fermentation pathways and an increased growth yield or substrate degradation of the primary cultures (Latham and Wolin, 1977, Teunissen et al., 1992, Williams et al., 1994). From these experiments it was concluded that hydrogen accumulation negatively affects fermentation, especially fibre degradation. However the results of later experiments were not so clear about the positive effects of hydrogen removal on growth and end products formation (Min et al., 2006). A different approach was to determine the effect of molecular hydrogen on fermentation in vitro. Work carried out with lactate indicated that lactate fermentation under a H_2 atmosphere lead to increased production of propionate at the expense of acetate, but at the same time the total substrate conversion was increased compared to a pure N_2 atmosphere (Schulman and Valentino, 1976).

However all these studies have been carried out in closed in vitro system where the dissolved hydrogen concentration can be 700 µM or higher in pressurised in vitro systems. The H_2 concentration in ruminants has been measured very rarely but generally is as low as 1 µM in an uninhibited rumen and can be as high as 50 µM after feeding (Smolenski and Robinson, 1988, Janssen, 2010). When methanogens are inhibited partially values as high as 100 µM are observed (J. Guyader, personal communication). However these values are much lower than the once expected in vitro and the aim of your study was to examine whether the hydrogen in the rumen of an animal affects, fibre digestibility, fermentation products or microbial community composition.

The methane yield in the control animals varied across the periods from 25.7 to 29.9 g/kg DMI. These values are relatively high compared to the current NZ inventory value of 21.6 g/kg DMI and data of pasture fed animals (van Zijderveld et al., 2010, Sun et al., 2012b) but well within the range of a dried 40% concentrate diet (Chong et al., 2013). The effect of the inhibitors to methane emissions was immediate and as a short term response H_2 was observed for a week. This is in contrast to work carried out with AQ in sheep where H_2 emissions were elevated during the whole 17 day experiment (Kung Jr et al., 2003). The acetate/propionate ratio in our experiment was similar to the ratio observed in cattle when methane emissions were inhibited with chloroform (Knight et al., 2011). Acetate / propionate ratio remained constant independent of the level of H_2 present or the level of methane inhibition. In an experiment with lambs (Trei et al., 1972) showed that in response to methane inhibition in the short term propionate is increased but over 90 days this shifts from propionate to increased

butyrate. However other than in our experiment these authors observed high hydrogen production (up to 20% of the rumen headspace) right until the end of the 90 day experiment. Using the same inhibitor (hemiacetal of chloral) in a later study showed hydrogen accumulation in lambs mainly during the first few days and much less after 4 weeks (Johnson, 1974). These results are similar to the results observed in the present study.
From some of the samples we were able to do a formate analysis, which showed that at the beginning of the inhibitor period formate was up to 20 mM and still at 6 mM at the end of the treatment period.
Digestibility of the dry matter (DMD), neutral and acid detergent fibre were determined at the end of each of the measurement periods. Therefore at the time when the digestibility was determined during the inhibitor period, only very little H_2 was observed. However yH in both the combination (0.67 g/kg DMI) and sequence (0.53 g/kg DMI) treatment were still higher (P=0.010) than the control (0.06 g/kg DMI). Apparent DMD of the treatments was similar to the control group during all of the three periods, but decreased in the recovery period compared to the baseline and treatment periods. Fibre content in this period tended to be lower but no significant differences in any of the chemical composition parameters was observed (data not shown). Similar results were observed in goats where methane inhibition with bromochlormethane did not affect DMD but a numerical increase in NDF digestibility was observed in these animals (Mitsumori et al., 2012). The effect on NDF and ADF digestibility was more profound in our experiments, where a significant increase in both, ADF and NDF digestibility was observed. However based on the data we conclude that methane inhibition and the slightly increased hydrogen concentration did not negatively influence ruminal fibre digestibility. In sheep methane inhibition with chloralhydrate did not have any effect on DMD (Mathers and Miller, 1982) and sheep fed BCM at 1.5 and 4.5 mg/kg BW showed a higher DMD compared to the controls (Sawyer et al., 1974). In fact there is very little evidence that methane inhibition has any negative effects on DMI or feed digestibility. In lambs the digestibility was increased when methane was inhibited with no effect on intake (Johnson, 1974). In another experiment using a hemiacetal of chloral and starch in a 90 day dose response trial (Trei et al., 1972) found positive effects of methane inhibition on live weight gain in lambs despite having high molecular hydrogen concentrations throughout the experiment.
The methanogen community composition was dominated by Methanobrevibacter gottschalkii and virtually no sequences of the classes Methanomicrobium, Methanosarcina or Methanobacter were observed. Overall the methanogen community of the sheep appears to be similar to the clades observed in lambs from Europe, Venezuela and Australia (Wright et al., 2004, Wright et al., 2008, Popova et al., 2013) where the Methanobrevibacter gottschalkii, smithii, millerae and thauri clade dominates. In the present experiment the least dominant group in the sheep was Methanosphaera species which contributed only 3% to the total methanogens. Thermoplasmatales (formerly rumen cluster C) and Methanobrevibacter ruminantium both made only around 10% of the total community in the control animals. When inhibitors were fed to the animals, the proportion of M. gottschalkii decreased to less than 5% as did M. ruminantium and the Methanosphaera species. One day after the first application of the inhibitors the Thermoplasmatales made up more than 90% of the total methanogen community. Thermoplasmatales have been shown to be the dominant rumen methanogens in Angus heifers that had been transitioned from pasture to a high grain diet (Tymensen et al., 2012). After a week however the proportion of Thermoplasmatales and M. ruminantium were equal and over the next few weeks M. gottschalkii partly recovered. At the end of the inhibitor period M. ruminatium was the dominant methanogen species present and the proportion of Thermoplasmatales was just slightly higher than in the control animals. Our results indicate that M. gottschalkii is most sensitive to the inhibitors and the least sensitive organisms are the Thermoplasmatales. In some pure culture work M. ruminantium showed the highest sensitivity to a whole range to methanogen inhibitors, while Methanomicrobium and

Methanosarcina species were more tolerant because of their ability to synthesise CoM (Ungerfeld et al., 2004). However none of these species appeared in the inhibited rumen. Differential tolerance of individual groups or species of methanogens to the treatments could explain the succession of methanogens observed in our experiment. Thermoplasmatales are methylotroph methanogens and more specifically can utilise methylamines (Poulsen et al., 2013), but it is not clear whether the methane free environment favoured the formation of methylamine from CO_2 and NH_4 in the rumen (Itabashi and kandatsu, 1978) or trimethylamine from choline (Neill et al., 1978).

The response in the bacterial community to methane inhibition was not as clear as for the methanogens. However the control group was significantly different ($P < 0.001$) from the two treatment groups and when a canonical discriminant analysis was used even the two treatment groups were separated. The main groups of organisms associated with the inhibitor treatment were Oscillospira a not well characterised member of the Ruminococcacea family, a unnamed genus in the Veillonellaceae which made up the largest genus (3.4%) and the genus Succinovibrio of which the main fermentation products are acetate and succinate but can also produce formate and lactate (rumen microbial ecosystem REF). The increased formate concentration observed could be mainly due to this organism but its overall proportion in the ecosystem is less than 1%.

Veillonellaceae are involved in lactate metabolism which other than butyrate and acetate metabolism does not release hydrogen. However, in the rumen lactate is quickly converted into acetate or butyrate (Marounek et al., 1989). Increased propionate production however is observed when H_2 concentrations are high (Schulman and Valentino, 1976).

Succinivibrio was decreased below the detection limit by the increased concentrate feeding (Tajima et al., 2001) but in a recent experiment with a methane inhibitor in cashew nut shell liquid this group of organisms was increased when methane was inhibited (Shinkai et al., 2012). However, Succinivibrio has a requirement for 1,4-naphthoquinone (Gomez-Alarcon et al., 1982), which is a precursor for the synthesis of 9,10-anthraquinone, one of the inhibitors used in this experiment, and therefore a contamination or the degradation of the inhibitor could have contributed to the observed increase in Succinivibrios. Very limited information on methane inhibition and its effect on bacterial community is available.

The main known bacterial fibre degrading bacteria tended to show as shift away from acetate producing Ruminococcus species towards more Butyriovibrio and Fibrobacter species which produced mainly butyrate and propionate and therefore produces less hydrogen per mole of glucose than the Ruminococci. Such a change in community composition would be expected but has not been shown in the work with Cashew shell liquid where all fibre degrading species were reduced while methane emission were decreased by 50%. It is however unknown whether the treatment was a general methane inhibitor which would increase hydrogen concentration or another inhibiting principle like a hydrogen sink.

The distance analysis revealed that the community in the baseline period was slightly closer related to the treatment period than the community in the recovery period in the control animals however overall the community in this group of animals was very similar. For both treatment groups the combination and the sequence of the inhibitors, the recovery period community was more closely related to the treatment community than the baseline community. This suggests that the treatment had a lasting effect, at least medium term on the microbial community.

CONCLUSION

The metabolic outcomes, ie. SCFA concentration and proportion were similar to the control animals after the treatments were stopped and no lasting effect was observed. The proposed negative effect of molecular hydrogen on fibre degradation as a consequence of methane inhibition was not observed. It is however unclear whether this is due to the fact that after a

10 day adaptation to the treatment only very little molecular hydrogen was released from the rumen or because of a lack of an effect of hydrogen on fibre degradation in vivo. Formic acid and propionic acid appear to be the dominant forms of hydrogen disposal on sheep when inhibited with chloroform and anthraquinone.

Objective 1, Milestones 7 & 8: Effect of methane inhibition on rumen function and growth of lambs.

BACKGROUND

The long term implications for rumen function in the absence of methanogens remain unknown. Increased hydrogen concentrations in the rumen in the absence of methanogens may inhibit fibre degradation and hence slow feed fermentation (McAllister and Newbold, 2008). Negative impacts on rumen function in the absence of methanogens will render technologies directly targeting methanogens less attractive unless carbon prices are high enough to offset any loss in productivity. However, most of the support for the hypothesis that hydrogen will decrease fermentation is derived from pure culture or *in vitro* work. The aim of this study was to examine the effect of methane inhibition on ruminal hydrogen concentration, microbiome composition and average daily gain in lambs.

MATERIALS AND METHODS

Experimental design and measurements

Twenty-four mixed sex lambs were randomly assigned to either a control group (no inhibitors) or a treatment group receiving methane inhibitors. The animals were adapted for 10 days to the experimental diet. During the adaptation and the subsequent 14 day baseline measurement, both groups were fed the control diet ad libitum with no inhibitors added. Methane was measured twice during the baseline period. After the baseline period the treatment group (n=12) received inhibitors in their diet, while no inhibitors were fed to the controls (n=12). Methane measurements were carried out every two weeks for six weeks. In addition to methane measurements, rumen fluid samples for metabolic and microbial community analysis were collected.

Animal handling

All manipulations were carried out with approval of the animal ethics committee at Grasslands, Palmerston North. The lambs were sourced from the Grasslands Animal Facility flock when they were 6-7 months of age and were 38.9 ± 1.4 kg at the start of the trial. The lambs were housed indoors in individual pens (1.5 x 2.5 m) on grated plastic flooring. Water was available *ad libitum* and feed was offered in two equal meals at 0800 h and 1600 h. Dry matter intake was determined daily from the weight difference of the feed offered and refusals. Methane measurements were carried out for two consecutive days in the sheep respiration chambers (Pinares-Patiño et al., 2008). At the end of each methane measurement period, rumen samples were collected via stomach tubing. The lambs were weighed every week before afternoon feeding.

Diet

The diet was made from chaffed hay, crushed barley, soy bean meal, molasses and a mineral mix at 300, 290, 300, 100 and 10 g/kg dry matter respectively. The hay was sourced form Wenham Grain & Seed, Palmerston North and the other ingredients from Denver Stock Feeds, Palmerston North. The chemical composition of the diet was 12 g/kg ash, 12 g/kg lipids, 133 g/kg crude protein, 286 g/kg neutral detergent fibre (NDF), 140 g/kg acid detergent fibre and 22 g/kg lignin (Massey University Nutrition lab, Palmerston North).

The inhibitors used in this study were 9,10-anthraquinone (AQ) and chloroform (CF) (Sigma, Auckland, New Zealand). Chloroform was complexed with cyclodextrin to create an odourless solid. The AQ and CF were added at 500 and 50 mg/kg of the diet respectively. Batches of diet containing inhibitors were prepared twice a week and stored in a cold room at 4 °C until used.

Rumen sampling and storage

Rumen sampling was carried out via stomach tubing after the lambs were removed from the respiration chambers. Rumen samples were subsampled for short chain fatty acid (SCFA) and ammonia (NH_4) analysis (1.8 ml), DNA extraction (0.9 ml), metabolomic analysis (1.8 ml) and RNA extraction (0.9 ml). DNA and RNA samples were snap-frozen and stored at -20 and -80 °C respectively. Samples for SCFA and metabolomic analysis were centrifuged (20,000 g, 10 min, 4 °C). An aliquot of 0.9 ml of the supernatant was collected into 0.1 ml of internal standard (19.8 mM ethylbutyrate in 20% v/v phosphoric acid) for SCFA and NH_4 analysis and stored at -20 °C. The remaining supernatant was collected for metabolomic analysis and frozen at -80 °C. The RNA and metabolomics samples have been archived for future analysis.

Analysis

SCFA samples were thawed and centrifuged (20,000 g, 10 min, 4 °C) and 800 µl of the supernatant was collected into a crimp cap glass vial. SCFA were analysed by a HP 6890 gas chromatograph (Attwood et al., 1998). The remaining supernatant was collected for ammonia analysis (Weatherburn, 1967).
DNA was extracted using a phenol chloroform method with a column clean-up of the DNA (Kittelmann et al., 2013) and primers targeting the microbial groups Archaea, Bacteria and Eukarya were used to amplify products for barcoded 454 sequencing (Kittelmann et al., 2013).
Data was analysed by analysis of variance (Genstat v16.2, VSN International, Hemel, Hempstead, UK), where the fixed effect was the inhibitor treatment vs. no inhibitor. $P<0.05$ was considered significant. Microbial community data were analysed using a principal coordinate analyses of Bray-Curtis dissimilarities and a PERMANOVA in combination with PERMDISP to determine significant differences between categories using QIIME (Caporaso et al., 2010).

RESULTS AND DISCUSSION

Dry matter intake and growth data

Dry matter intake and live weight is shown in (Figure 1). Dry matter intake decreased 1-2 days after the start of the inhibitor treatment. During the treatment period the treated animals had reduced DMI maintained body weight. We observed that the animals that received the inhibitors preferentially selected for the chaffed hay particles in the diet, thereby selecting against the inhibitors. After four weeks the treatment, the animals still consumed only half of their allocated diet and the experiment was terminated. In order to establish whether the lambs could recover once the inhibitors had been removed from the diet, all lambs were fed the control diet for another three weeks and their DMI was monitored daily. Three to four days after the inhibitors were removed, DMI of the treatment group increased (Figure 1a) and the animals gained more weight (304 g/d) than the control animals (192 g/d) during the three week recovery period.

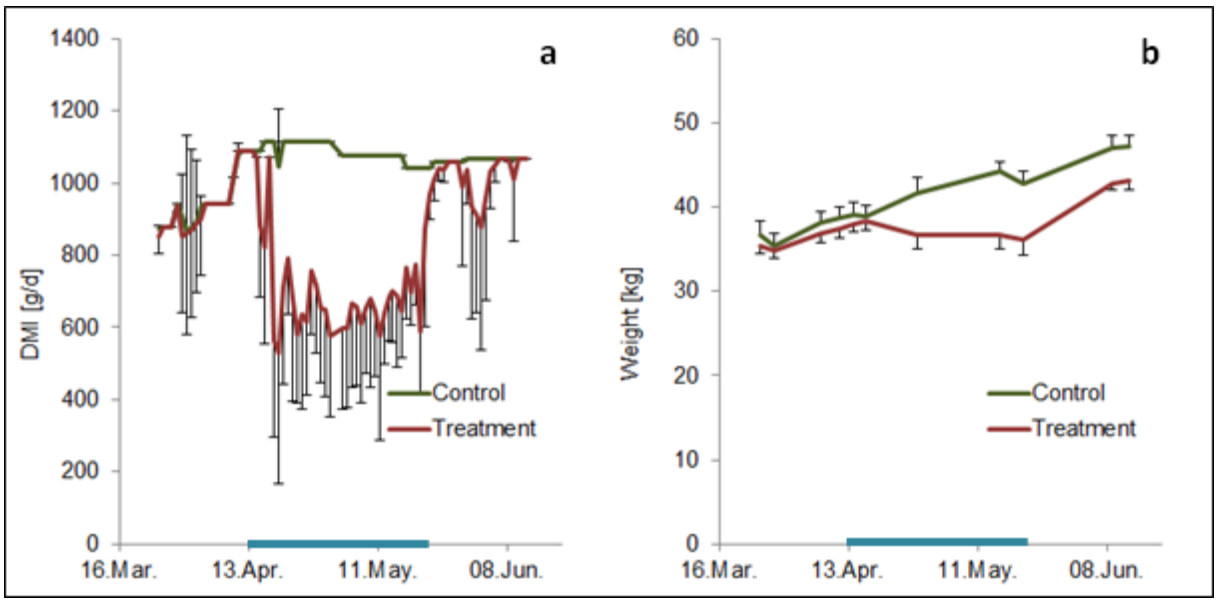

Figure 1. Dry matter intake (DMI) and live weight of the lambs in response to methane inhibition (blue bar) and removal of the inhibitors.

Methane and hydrogen emissions

Methane production was decreased when the two inhibitors were fed to the lambs (Figure 2a). However, most of this effect was due to the decreased DMI of the treatment group (Figure 1a). The effect on methane emissions was less pronounced when methane was expressed per kg dry matter eaten (Figure 2b). Three weeks after the inhibitors were removed from the diet, the DMI of the two groups was similar (Table 1) but the methane production remained reduced in the treatment group compared to controls and therefore decreased methane yield (Table 1). However, in the previous experiment with lambs (Milestones 2 & 4) the methane yield 5 weeks after the removal of the inhibitors from the diet was similar to the values of the control group. Overall, the methane yield in this experiment was higher when compared to ryegrass-fed sheep (Hammond et al., 2011, Sun et al., 2012a). Compared the to the control animals in the previous lamb experiment (Milestone 2 & 4), methane yield of the control lambs was lower, which could partly be due to the higher amounts of crushed barley used in the present experiment leading to a lower pH and lower methane emissions (Janssen, 2010). The effect of methane inhibition on hydrogen emissions was relatively small, as hydrogen explained less than 30% of the methane reduction.

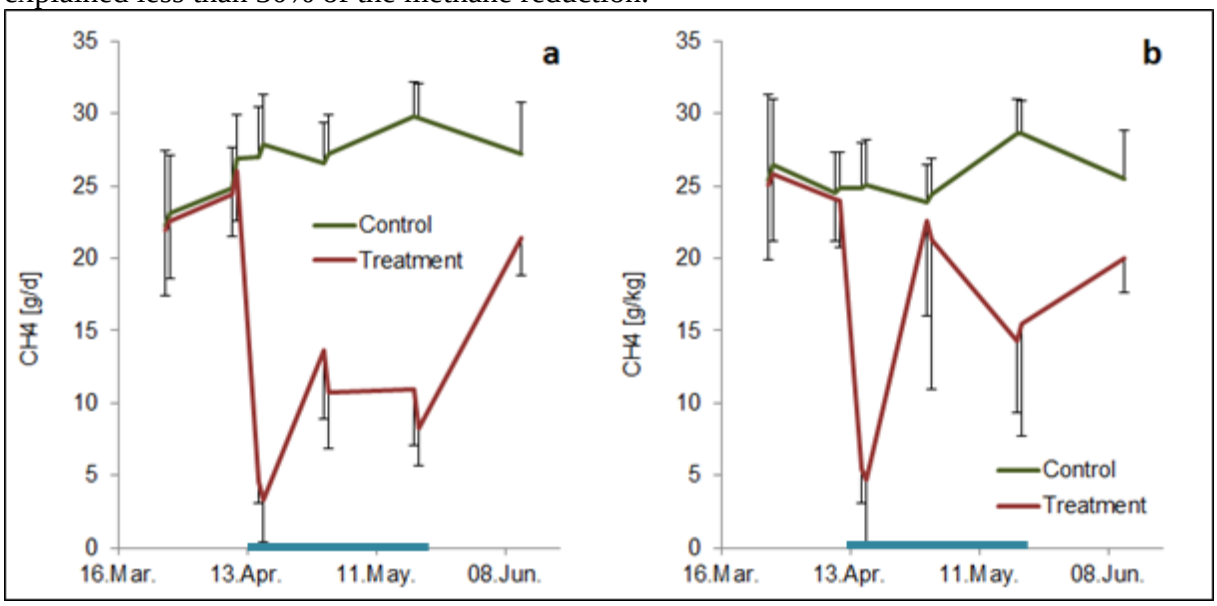

Figure 2. Methane (CH4) production (a) and yield (b) of the lambs in response to methane inhibitors offered in the diet (blue bar).

Table 1. Difference in dry matter intake (DMI), methane (CH4) and hydrogen (H2) yield between the control and treatment (inhibitor) groups.

Parameter	Period	Control	Treatment	SED	P value
DMI [kg/d]	Baseline	0.96	0.96	0.019	0.912
	Inhibitor	1.08	0.72	0.028	<.001
	Recovery	1.07	1.07	0.000	1.000
CH_4 [g/kg]	Baseline	25.3	24.7	0.83	0.486
	Inhibitor	25.9	13.9	1.19	<.001
	Recovery	26.0	20.1	1.04	<.001
H_2 [g/kg]	Baseline	1.3	1.1	0.082	0.202
	Inhibitor	1.0	3.6	0.241	<.001
	Recovery	1.0	1.2	0.076	0.009

Rumen metabolite profile

Ruminal SCFA concentration was similar between the control and the treatment group during the baseline, treatment and the recovery periods (Table 2). During the treatment period, the proportion of acetate was decreased in the treated lambs, while both propionate and butyrate proportion was increased compared to controls. This is expected as propionate and butyrate pathways consume hydrogen (Janssen, 2010) and hydrogen concentration was elevated during the treatment period (Table 1). During the recovery period, the differences in SCFA composition were lower but the treatment animals still had lower acetate and a higher propionate proportion in the rumen compared to controls. This result is consistent with the lower methane yield of the animals during that time. No effect of the inhibitors on the minor fatty acids were observed.

Table 2. Effect of methane inhibition in the rumen short chain fatty acid (SCFA) concentration and composition.

Parameter	Period	Control	Treatment	SED	P value
SCFA [mM]	Baseline	49.3	49.6	2.74	0.907
	Inhibitor	55.2	54.8	3.42	0.926
	Recovery	54.6	48.5	4.58	0.192
Acetate [%]	Baseline	64.0	63.7	0.91	0.713
	Inhibitor	63.3	53.5	0.85	<.001
	Recovery	63.3	59.7	1.44	0.020
Propionate [%]	Baseline	15.0	15.3	1.04	0.788
	Inhibitor	14.9	21.4	1.01	<.001
	Recovery	14.9	19.6	1.88	0.020
Butyrate [%]	Baseline	13.6	13.9	0.78	0.643
	Inhibitor	14.8	18.3	0.76	<.001
	Recovery	14.7	13.0	1.17	0.162
Minor [%]	Baseline	5.2	5.0	0.39	0.510
	Inhibitor	5.0	4.6	0.23	0.065
	Recovery	4.9	5.3	0.48	0.469

Microbial community composition

The *Methanobrevibacter gottschalkii* clade which in an uninhibited rumen dominates the methanogen community comprising 70% of the methanogen community and is the only methanogen group that was negatively affected when the inhibitors were introduced (Figure 3). The Thermoplasmatales methanogen community increased as an initial response to the inhibitors followed by an increase in the proportion of Methanobrevibacter ruminantium . When the inhibitors were removed from the diet, Mbb gottschalkii recovered to the pre-treatment proportion. This sequence of events of has also been shown in the first inhibitor trial in sheep (Milestones 2 & 4, June 2014). Although lower methane emissions were observed in the treatment group during the recovery period compared to the baseline period, the community composition at the species level of methanogens was similar between the baseline and recovery period (Figure 4b).

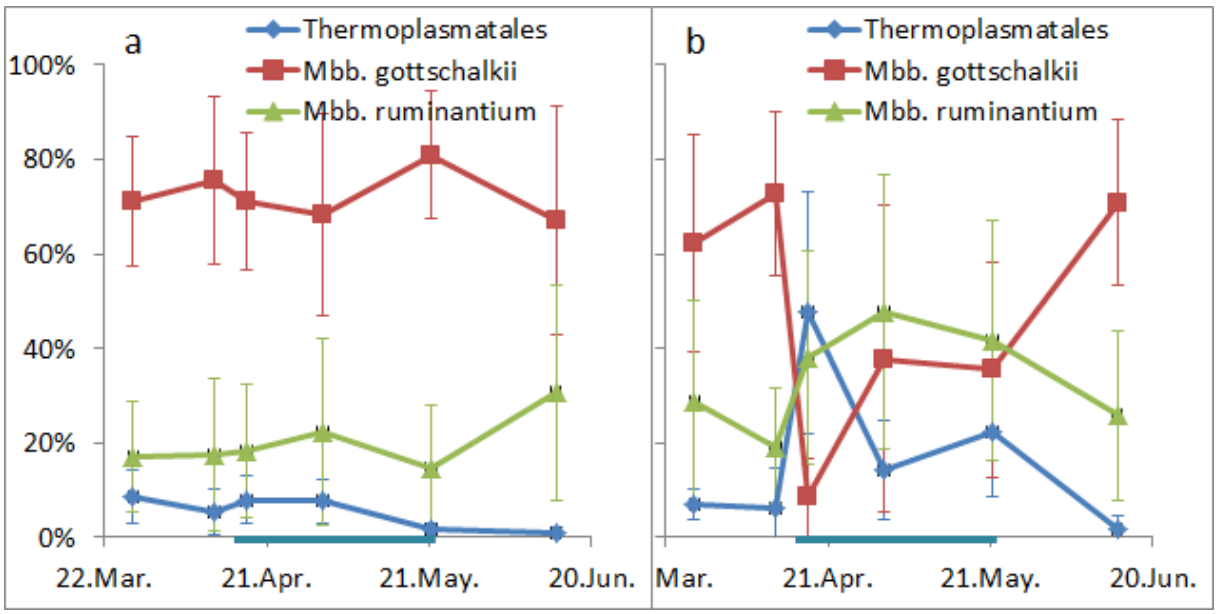

Figure 3. Methanogen community composition in the control group (a) and the treatment group (b) before, during (blue bar) and after the treatment with inhibitors.

Methane inhibitors also affected the bacterial community ($P < 0.0001$) significant (Figure 4a), but other than in methanogens the effects persisted into the recovery period. The bacterial composition of the treatment group was similar between the treatment and recovery period ($P = 0.624$). However, the during the recovery period the bacterial communities of the control and treatment group only tended to be different ($P = 0.059$). This result is in agreement with the differences in SCFA composition that were observed for the recovery period.
Inhibition of methanogens did not affect the composition of the protozoal community in the rumen although close spatial and metabolic associations between methanogens and protozoa have been shown (Finlay et al., 1994, Tokura et al., 1997). In this experiment the differences in the protozoal population appeared to be related to a change in protozoal community over time (Figure 4 C) and differences were observed between the baseline and the recovery period for both the control ($P = 0.008$) and the treatment group ($P = 0.003$).

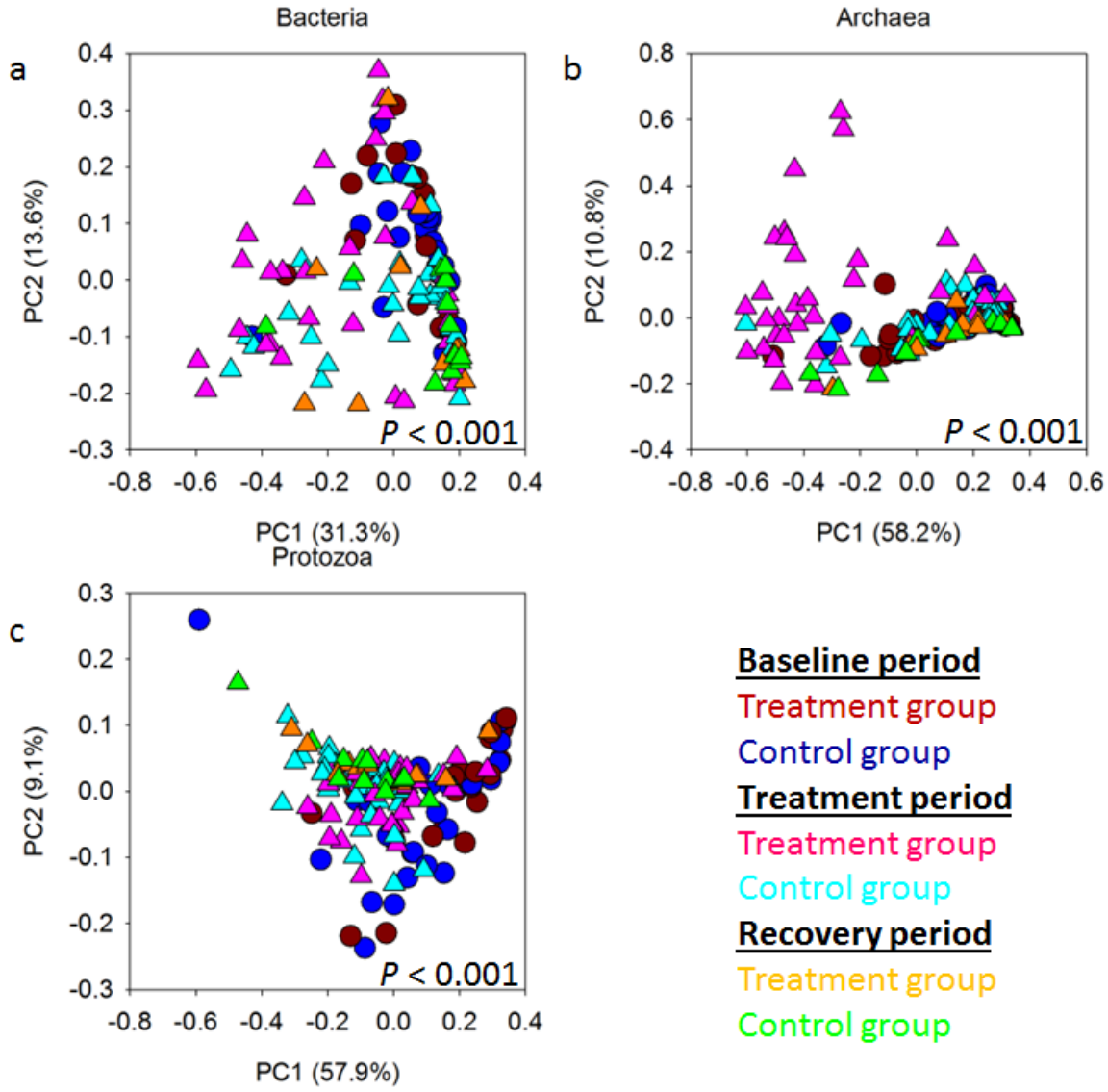

Figure 4. Principal coordinate analyses of Bray-Curtis dissimilarities of rumen A) bacterial, B) archaeal, and C) protozoal microbial community compositions in sheep receiving either methane inhibitors (▲) or a no inhibitor control (●).

CONCLUSION

Feeding chloroform and 9,10-anthraquinone appear to decrease DMI of lambs and consequently negatively affect live weight gain. Based on this experiment we cannot differentiate whether this negative effect on DMI is due the ruminal hydrogen or due to a direct effect of one of the inhibitors on the animal itself. The observation that the hydrogen concentration was comparatively low (although significantly higher than in the controls) would indicate that the negative effect on DMI was driven by a direct effect rather than a negative effect of ruminal hydrogen on fermentation. A comparison of the experiments in sheep and cattle will hopefully help to identify the cause for the decrease in performance. This comparison will be one focus in the final report and the topic of a presentation that we plan to present at the next greenhouse gas in animal agriculture (GGAA) conference.

Objective 1, Milestone 9: Effect of methane inhibitors on milk production in dairy cattle

BACKGROUND

The long term implications for rumen function in the absence of methanogens remain unknown. Increased hydrogen concentrations in the rumen in the absence of methanogens may inhibit fibre degradation and hence slow feed fermentation (McAllister and Newbold, 2008). Negative impacts on rumen function in the absence of methanogens will render technologies directly targeting methanogens less attractive unless carbon prices are high enough to offset any loss in productivity. However, most of the support for the hypothesis that hydrogen will decrease fermentation is derived from pure culture or *in vitro* techniques, which are closed systems, and hydrogen pressure can be very high. The aim of this study was to examine the effect of methane inhibition on ruminal hydrogen concentration, fermentation parameters and milk production in dairy cattle.

MATERIALS AND METHODS

Experimental design and measurements

All manipulations were carried out with approval of the animal ethics committee at Grasslands, Palmerston North (AE13256). Sixteen dairy cows were randomly assigned to either a control group (no inhibitors) or a treatment group receiving methane inhibitors (9,10-anthraquionone and chloroform) mixed into the total mixed ration (TMR) diet. The animals came from pasture and were first adapted for 7 days to the TMR with no inhibitors added. After this diet adaptation period, the treatment group received inhibitors added to the diet for 24 days before methane was measured. During the first 10 days, only half of the final concentration of inhibitors was added to the TMR of the treatment group to adapt the animals slowly to the treatments. The full dose of the inhibitors was offered to the animals for 2 weeks before methane measurements were carried out.

Diet and inhibitors

The TMR was formulated from chaffed hay, crushed barley, soy bean meal, molasses and a mineral mix at 300, 290, 300, 100 and 10 g/kg dry matter (DM) respectively. The hay was sourced from Wenham Grain & Seed, Palmerston North and the other ingredients from Denver Stock Feeds, Palmerston North. The chemical composition of the TMR was 12 g/kg ash, 18 g/kg lipids, 163 g/kg crude protein, 286 g/kg neutral detergent fibre (NDF), 140 g/kg acid detergent fibre and 22 g/kg lignin. The wet chemistry analysis was carried out by the Nutrition Lab, Massey University, Palmerston North.
The inhibitors used in this study were 9,10-anthraquinone (AQ) and chloroform (CF) (Sigma, Auckland, New Zealand). Chloroform was complexed with cyclodextrin to create an odourless solid. The AQ and CF were added at 500 and 50 mg/kg of the diet respectively. Batches of diet containing inhibitors were prepared twice a week and stored in a cold room at 4 °C until used.

Animal handling

The 16 dairy cows were selected from Dairy No1, Massey University Palmerston North. The average weight of the cows was 501 ± 37 kg. The cows were multiparous spring calving Friesian x Jersey cows that were between 3 and 11 years old. During adaptation, the cows were group fed their diet on a feed pad covered with wood chips. Water was available *ad*

libitum and the TMR was offered in two equal meals at 0900 h and 1600 h. The cows were milked once a day at 0800 h. Group feed intake was measured from the weight difference of feed offed and refused from the feed pad. Rumen samples were collected via stomach tubing before the inhibitor treatment started and 2 weeks into the adaptation period. The last sample was collected after the methane measurements were carried out.
The cows were brought to Grasslands 3 to 4 days before the methane measurements to give them time to adapt to the surroundings and the confinement. The cows were milked once a day in the morning at 0830 h and milk volume was determined gravimetrically during adaptation and while in the chambers. Milk composition was determined by LIC (Hamilton, NZ) using infrared spectroscopy. Milk production (kg/d) and milk yield (kg/kg DMI) were calculated. After the acclimatisation period, methane emissions were measured for 2 consecutive days using open circuit respiration chambers as described in Chapter 1 of the technical manual on respiration chamber design (Pinares et al., 2012; http://www.livestockemissions.net/reports, listing,73,technical-manual-on-respiration-chamber-designs.html). Dry matter intake (DMI, kg/d) was recorded during methane measurements from the weight difference of feed offered and refused.

Sample analysis

Rumen samples from stomach tubing were immediately subsampled for short chain fatty acid (SCFA) analysis (1.8 ml). The samples were centrifuged (20,000 g, 10 min, 4 °C). An aliquot of 0.9 ml of the supernatant was collected into 0.1 ml of internal standard (19.8 mM ethylbutyrate in 20% v/v phosphoric acid) for SCFA analysis and stored at -20 °C.
SCFA samples were thawed and centrifuged (20,000 g, 10 min, 4 °C) and 800 µl of the supernatant was collected into a crimp cap glass vial. SCFA were analysed by a HP 6890 gas chromatograph (Attwood et al., 1998).

Statistical analysis

Data was analysed by ANOVA (Genstat v16.2, VSN International, Hemel, Hempstead, UK), where the fixed effect was the inhibitor treatment vs. no inhibitor. $P<0.05$ was considered significant.

RESULTS

Prior to the experiment, the cows were on pasture, but they adapted quickly to a TMR and were consumed up to 18 kg TMR dry matter per day during adaptation period. However, when the inhibitors were introduced to the diet a change in feeding behaviour between the treatment and control group was observed. The cows receiving the inhibitor TMR had a slower rate of feed intake than those receiving TMR without inhibitors. The treatment cows consumed their diet allocation over the whole day, while the control animals consumed the feed offer within an hour. The intake of cows receiving TMR with inhibitors reduced their feed intake by 30% during the first week compared with those receiving TMR without inhibitor. Over the next 3 to 5 days the treatment group intake increased again to 80 – 90 % of the control group's consumption.
During the methane measurement period the DMI of the treated animals was 20 % lower (Table 6) compared to the control animals. The inhibitors decreased methane yield by 59% and significant amounts of molecular hydrogen emissions (H_2) were observed (Table 6). An average 16 moles of hydrogen were emitted from each treated cow per day. Only 0.02 moles of hydrogen were observed in the control animals. However, this amount of hydrogen explained only one third of the hydrogen that is not captured in methane in the treated animals.
Milk production in cows fed TMR containing the inhibitors tended to be lower than those in control group. However, the milk yield was similar (Table 1). No difference in milk fat and

lactose content was observed. However, protein content in the milk of the treatment cows was increased (Table 1).

Table 6 Dry matter intake (DMI), methane (CH_4) and hydrogen (H_2) emissions, milk production and composition in dairy cows fed methane inhibitors chloroform and anthraquinone (CF/AQ) compared to a control

Parameter	Control	CF/AQ	sed	P value
DMI [kg/d]	16.5	13.2	0.87	0.002
CH_4 yield [g/kg DMI]	22.3	9.2	1.50	<.001
H_2 yield [g/kg DMI]	0.0	2.5	0.57	<.001
Milk production [kg/d]	15.2	13.1	1.06	0.069
Milk yield [kg/kg DMI]	0.92	0.99	0.06	0.232
Milk fat [g/kg]	50.3	45.6	3.56	0.204
Milk lactose [g/kg]	48.8	48.9	0.95	0.914
Milk protein [g/kg]	40.5	43.9	1.46	0.020

The methane inhibitors also changed the rumen fermentation profiles (Table 2). Total SCFA concentration in the rumen was higher in the cows receiving the inhibitors and proportion of the both the proportions of propionate and butyrate were increased at the expense of acetate.

Table 7 Short chain fatty acid (SCFA) concentration and fermentation profile in dairy cows fed methane inhibitors chloroform and anthraquinone (CF/AQ) compared to a control

Parameter	Control	CF/AQ	sed	P value
SCFA [mM]	53.5	61.3	3.70	0.033
Acetate [%]	68.3	58.5	0.74	<.001
Propionate [%]	14.3	18.7	0.66	<.001
Butyrate [%]	11.3	14.5	0.82	<.001

DISCUSSION

With the current data we cannot fully exclude a negative effect of ruminal hydrogen on DMI and consequently on milk production. Based on the results observed in calves we hypothesise that the reduced intake observed in dairy cattle is due to a direct effect of either the chloroform or the anthraquinone on the metabolism of the animal or palatability of the diet. In calves, even higher hydrogen concentrations were observed in the rumen (Milestone reports 3 and 5) and no negative effects of the same inhibitor concentration on DMI or intake behaviour was observed. In addition, some recent results with 3-nitrooxypropanol (3NP) have shown that methane inhibition did not reduce DMI or milk production (Reynolds et al., 2014). Using 3NP as an inhibitor, the cows also had an increased milk protein content and increased ruminal concentrations of propionate at the expense of acetate. However, compared to our experiment, the reduction in methane emissions achieved by 3NP was comparatively small. In another study, chloroform dosed to dairy cows did not result in any negative effects on live weight, DM digestibility, rumen fill and retention time, suggesting no negative effect on rumen fermentation (Knight et al., 2011). We therefore hypothesise that the negative effects on DMI in our experiment are related to possible effects AQ on diet palatability.
Ruminal propionate or amino acids, mainly glutamates, are the main substrate for gluconeogenesis in the ruminant (Aschenbach et al., 2010). The high propionate production in cows receiving the inhibitors may lead to less demand for amino acids for gluconeogenesis and therefore could have contributed to the higher milk protein content in the cows receiving the inhibitors. The higher concentration of SCFA in the rumen of the treatment cows might be a consequence of their feeding behaviour, since rumen fluid was collected after milking but

before the morning feed allowance. The lower rate of intake in the treatment cows would have resulted in a higher concentration of SCFA, since these cows have more undigested feed in the rumen. Acetate and butyrate are both lipogenic fatty acids, that are used in the animal either for energy metabolism or to synthesise fatty acids.

Although some negative effects of methane inhibition on DMI and milk production was observed, the overall results from recent work overseas combined with the calf trials carried out within this programme, we conclude that increased hydrogen emissions resulting from methane inhibition does not drive the negative effect on rumen fermentation and production, but rather that the negative effects observed on DMI are due to effects of the AQ on palatability.

Objective 2, Milestone 3 & 6: Effect of early life inhibitor intervention on methane emissions later in life

INTRODUCTION

Decreasing methane emissions form ruminants could be one alternative to decrease the total greenhouse gas emission in New Zealand since around 1/3 of the carbon dioxide equivalents stem from enteric fermentation. For the New Zealand pastoral farming system simple feed or feed additive solutions are not applicable and different approaches from genetic selection fro low methane ruminants to attempts to immunise against methane producing microorganisms are trialled. Immunisation is one form of modifying the rumen microbiome. From previous work on rumen function it is known that there are large individual differences in the microbial community of ruminants of the same species, but it is virtually impossible to change the makeup of the community in an adult ruminant (Klieve et al., 2012). However, it has been shown that early life interventions, when the rumen community is developing, can lead to persistent changes in the microbial community composition in goats (Abecia et al., 2013, Abecia et al., 2014). Such a short term early life intervention could lead to a positive a low methane rumen by leaving an imprint in the microbial community. All these changes in rumen function however, must not decrease animal productivity.
In this pilot study, methanogen inhibitors were applied for 5 and 10 weeks post-partum to establish whether an early life intervention during rumen microbial community development, would lead to lower methane emission after the inhibitor treatments stopped at 10 weeks of age.

MATERIAL AND METHODS

Experimental design

Twelve bull calves were randomly assigned to one of three treatment groups at 4 days of age. Methane inhibitors were administered for a period of 5 weeks (T5), 10 weeks (T10) and compared to an untreated control group (Co).
Methane measurements were carried out every two weeks prior to weaning at 10 weeks, and in 4 week intervals after weaning until 24 weeks of age. A total of 8 methane measurements were carried out during these 24 weeks and samples of rumen contents were collected after every methane measurement.

Measurements

Calf live weight, methane emissions, dry matter intake and end products of rumen fermentation were determined.

Animal handling

The calves were purchased from Massey University, Dairy No. 1 at 3-4 days of age. While the animals were still at the farm, they were allowed access to colostrum. The calves were then transferred to the Animal Facility at AgResearch Grasslands, where they were housed indoors in individual pens. During the first 10 weeks of life, the calves were offered calf milk replacer (Miligans, Omaru, NZ) in addition to a started meal (Table 8). Starting from week 4 a hay-based general purpose diet (GP; Table 8) was offered in addition to the starter meal, Intake of milk replacer, starter meal and GP was determined on a daily basis. The animals received 2L of milk in the morning and the same amount in the afternoon. Water was available *ad libitum*.

Methane measurements were carried out for 24 h in the cattle respiration chambers with the air flow decreased to 600 L/min to account for the lower methane production rates of a young animal. Solid feed was offered in the morning in the chambers and water was available *ad libitum*. Milk was offered to the calves in the morning and in the afternoon as during the pen period.
After weaning from milk replacer at 10 weeks, the calves were group housed for 4 weeks in a pen indoors to enable weaning of starter meal and acclimatization to the GP diet offered *ad libitum*. After this period, the calves were transferred to an outdoor paddock with minimal pasture availability and were group-fed the GP diet *ad libitum*.
The calves were weighed weekly before morning feeding.

Diet

The composition of the starter meal and the GP diet is given in Table 8 and the chemical composition is shown in Table 9.

Table 8 Ingredients of the starter meal and the general purpose (GP) diet

Starter Meal	g/kg	**GP diet**	g/kg
Maize	108	Hay	500
Barley	432	Barley	290
Peas	173	Soya	100
Soya	205	Molasses	100
Molasses	54	Di-calcium-phosphate	5.5
Sodium bicarbonate	20	Salt	3.0
Salt	5.0	Mineral/vitamin mix	1.5
Calf pre-mix	1.0		
Bovatec	0.6		
Rumasweet palatant	0.2		

Table 9 Chemical composition (g/kg) of the starter meal and the general purpose diet

Diet	Milk replacer[1]	Starter Meal	GP diet
DM	970	887	828
Ash	62	59	56
Protein	227	208	133
Fat	206	17	11
NDF		155	438
Sugars	495*		51

1 Manufacturers data, * Lactose, NDF, Neutral detergent fibre, ADF acid detergent fibre

The inhibitors used in this study were 9,10-anthraquinone (AQ) and chloroform (CF) complexed with cyclodextrin. Concentrations were 500 and 50 mg/kg starter meal respectively. Both AQ and CF were mixed into 1/5 of the final amount of starter meal using a food processor and then mixed into the final batch using a commercial concrete mixer. Batches were prepared approximately every 4 days and stored in a cold room until used. Intake of starter meal therefore determined inhibitor intake.

Rumen sampling

Rumen sampling was carried out via stomach tubing after the calves were removed from the respiration chambers. Rumen samples were subsampled for short chain fatty acid (SCFA) and ammonia (NH_4) analysis (1.8 ml), DNA extraction (0.9 ml), metabolomic analysis (1.8 ml)

and RNA extraction (0.9 ml). DNA and RNA sampled were snap-frozen and stored at -20 and -80 °C respectively. Samples for SCFA and metabolomic analysis were centrifuged (20,000 g, 10 min, 4 °C) An aliquot of0.9 ml of the supernatant was collected into 0.1 ml of internal standard (19.8 mM ethylbutyrate in 20% v/v phosphoric acid) for SCFA and NH_4 analysis and stored at -20 °C. The remaining supernatant was collected for metabolomic analysis and frozen at -80 °C.

Analysis

SCFA samples were thawed and centrifuged (20,000 g, 10 min, 4 °C) and 800 µl of the supernatant were collected into a crimp cap glass vial. SCFA were analysed in a HP 6890 gas chromatograph [1]. The remaining supernatant was collected for ammonia analysis [2].
DNA extraction was carried out using a phenol-chloroform protocol combined with mechanical cell disruption (Rius et al., 2012). This method was modified slightly for DNA extraction from liquid samples as follows.

A subsample of 200 µl of whole rumen fluid was added to 200 µl 20% SDS, 182 µl buffer A (200 mM NaCl, 200 mM Tris, 20 mM EDTA, pH 8), 168 µl buffer PM (QIAquick 96 PCR purification kit, Qiagen, Valencia, CA, USA), 550 µl phenol-chloroform-isoamyl alcohol mixture (25:24:1, pH 8) and 0.7 g 0.1 mm zirconium beads. A BioSpec mini-beadbeater-96 (BioSpec Products Inc., Bartlesville, OK, USA) was employed for cell disruption at 2100 oscillations/min for 4 min. After centrifugation (20 min, 14 000 rpm, 4°C), 350ul of supernatant was mixed with 650 µl buffer PM (Qiagen) before transferring and binding DNA to the membrane of a Qiagen QIAquick 96-well plate. After washing bound DNA with buffer PE (Qiagen), elution was performed by the addition of 100 µl elution buffer (Qiagen) under vacuum. Eluted DNA was quantified using an Invitrogen Quant-iT dsDNA assay, broad range kit (Life Technologies, Carlsbad, CA, USA) and FlexStation 3 microplate reader fluorometer (Molecular Devices, Sunnyvale, CA, USA).

PCR amplification of bacterial and archaeal 16S rRNA genes, and ciliate protozoal 18S rRNA genes was carried out following a method by (Kittelmann et al., 2013). The primers used were synthesized by Integrated DNA Technologies Inc. (Coralville, IA, USA) and consisted of a 454 Life Science adaptor A (5'-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG-3') or B (5'-CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG-3') for titanium sequencing, a 2-base linker, a template specific sequence, and a unique Golay barcode attached to adaptor A (for unidirectional sequencing) for each sample, to allow for sample identification after amplicon pooling.

Triplicate PCR products from each microbial group for each sample were pooled and product size checked by agarose gel electrophoresis. Following the quantification of the pooled triplicates (using an Invitrogen Quant-iT dsDNA assay, broad range kit (Life Technologies) and FlexStation 3 microplate reader (Molecular Devices)), pooling of PCR products for each microbial group into separate vials was performed. Products were pooled in varying volumes to achieve 150 ng PCR product from each sample in the three vials: bacteria, fungi, and protozoa. The pooled vials were then quantified using Invitrogen Qubit dsDNA assay, high sensitivity kit (Life Technologies) and an Invitrogen Qubit fluorometer (Life Technologies). 1 µg of DNA from each pooled vial was loaded onto an agarose gel (1% wt/vol) and each band was excised under an Invitrogen Safe Imager 2.0 Blue-Light Transilluminator (Life Technologies). Each band was purified using a QIAquick gel extraction kit (Qiagen) and then quantified in triplicate using a Qubit dsDNA assay, high sensitivity kit (Invitrogen). The purified pools were diluted to $1x10^9$ molecules/µl and mixed at the ratio 5:1:1 bacteria:archea:protozoa PCR products. The products were then sent for 454 sequencing to MWG, Braunschweig, Germany.

.
Data analysed using in the Annova procedure (Genstat V13) comparing the effects of the treatments and effects of age of the calves. P values < 0.05 were declared significant.

RESULTS AND DISCUSSION

Throughout the experiment the calves had similar average daily gains (P = 0.584; Figure 8). We observed a decrease in DMI during the transition from the starter meal to the GP diet which is comparatively low in protein content. However, these issues were observed in all groups including the control and thus are not attributed to the methane inhibition treatment.

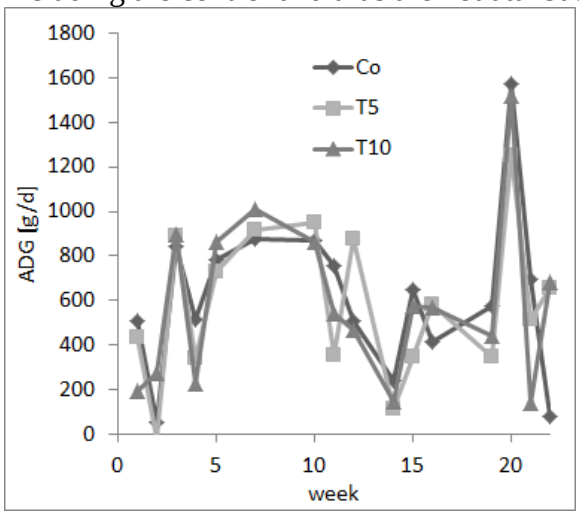

Figure 8 Weight of the treatment animals over a 20 week period

DMI increased with the nutritional demand of the animals. Over most of the experiment no differences in starter meal and GP diet intake were observed between the treatment groups (Table 10). Only in week 7 total DMI of the T10 group was higher that of the control and the T5 group. As shown in Figure 9 this difference was entirely driven by a high meal intake of the animals in group T10 while GP diet intake was similar at week 7.

Table 10 Dry matter intake [kg/d] of the calves during the course of the experiment.

Time	Co	T5	T10	sed	P value
Week 3	0.2	0.2	0.2	0.04	0.666
Week 5	0.6	0.5	0.6	0.11	0.560
Week 7	0.8	0.7	1.2	0.16	0.038
Week 10	2.0	2.1	2.0	0.41	0.930
Week 12	2.6	2.6	2.6	0.42	0.971
Week 15	2.5	2.2	2.4	0.22	0.615
Week 19	3.5	3.6	3.6	0.16	0.794
Week 23	4.3	4.3	4.3	0.09	0.505
Sed	0.29	0.23	0.20		
P value	<0.001	<0.001	<0.001		

sed, standard error of the differences;
grey shaded cells represent times when methane inhibitors were applied

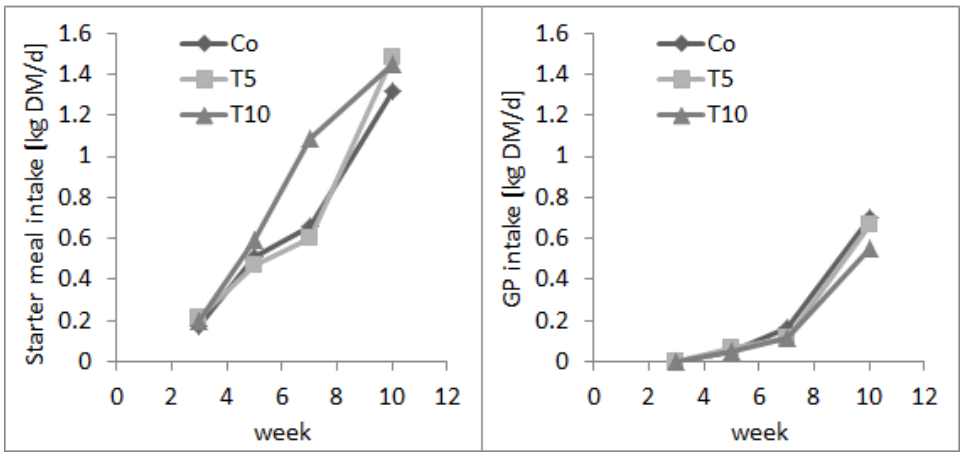

Figure 9 Starter meal and GP intake of the calves until weaning at week 10

Methane yield (g CH_4 / kg DMI) differed between the treatment groups only during the period when inhibitors were fed to the calves, i.e. until week 5 and 10 for the Groups T5 and T10 respectively (**Error! Reference source not found.**). No significant difference in the methane emissions during the first measurement (week 3) was observed which is likely due to low and variable DMI between animals and the overall low methane production of only 1.7 g/d in the uninhibited control group. The increased proportion of GP diet consumed by the T10 group did not affect the ability of the inhibitors added to that starter meal to inhibit nearly all methane inhibition at week 10. However within the treated animals variability of methane emissions was very high as indicated by the comparatively high error values indicating that the treatments affected individual calves to a different extent. Methane emissions of the starter meal resemble that of a typical high concentrate diet with values around 16 g/kg DMI and the values are well within the range observed in cattle fed high concentrate diets. When the diet was changed to the relatively high fibre GP diet methane yield increased significantly to values typical for roughages [4,5].

Table 11 Methane yield [g/kg DMI] of the calves during the course of the experiment

Time	Co	T5	T10	sed	P value
Week 3	11.6	3.4	5.9	4.56	0.236
Week 5	15.0	6.3	0.8	4.09	0.021
Week 7	17.0	20.3	3.0	6.24	0.048
Week 10	14.4	15.7	3.8	2.41	0.001
Week 12	23.1	19.8	19.3	2.62	0.309
Week 15	25.5	27.3	24.4	1.93	0.367
Week 19	20.4	20.9	20.2	1.40	0.858
Week 23	20.7	20.5	19.7	1.04	0.618
Sed	2.95	4.73	2.18		
P value	0.001	<0.001	<0.001		

sed, standard error of the differences;
grey shaded cells represent times when methane inhibitors were offered in the starter meal.

Molecular hydrogen was a prominent hydrogen disposal route in calves over during methanogen inhibition (Table 8). This contrasts with results obtained from sheep dosed with the same inhibitor combination, in which molecular hydrogen was only a transient product in response to methane inhibition. In the present experiment, large differences in hydrogen production were also observed between individual calves dosed with the inhibitors (Figure 10). In fact up to week 10, one of the treated calves in group T10 produced enough molecular hydrogen to explain more than 75% of the hydrogen calculated from the methane production of the control animals, assuming that for the production of 1 mole of methane 4 moles of hydrogen are required [6].

Table 12 Hydrogen production [g/d] of the calves during the course of the experiment

Time	Co	T5	T10	sed	P value
Week 3	0.2	0.1	0.5	0.20	0.173
Week 5	0.2	1.3	2.1	0.61	0.036
Week 7	0.1	0.3	3.6	1.97	0.195
Week 10	0.1	0.1	3.9	0.72	<0.001
Week 12	0.0	0.0	0.0	0.02	0.424
Week 15	0.0	0.0	0.0	0.01	0.120
Week 19	0.0	0.1	0.0	0.04	0.482
Week 23					
sed	0.05	0.26	1.41		
P value	0.003	<0.001	0.028		

sed, standard error of the differences;
grey shaded cells represent times when methane inhibitors were applied

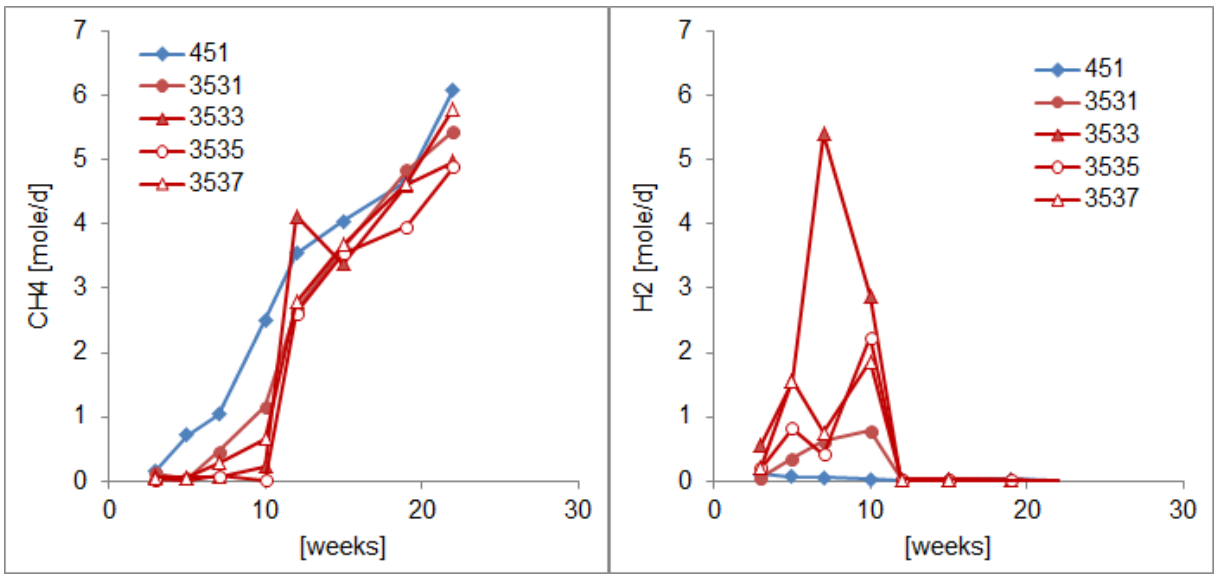

Figure 10 Methane and hydrogen emissions of a control calf (blue) and two treated calves.

Propionate was one of the major routes of hydrogen disposal when methane was inhibited in sheep[7] as indicated by the increase in the proportion of propionate in total ruminal SCFAs in sheep.[7] Such an increase in propionate in sheep in response to methane inhibition has also been observed in experiments with AQ [8]. In contrast, such a metabolic pathway shift from acetate (hydrogen producing pathway) to propionate (hydrogen consuming pathway) was not observed in calves (**Error! Reference source not found.**). Two different scenarios could lead to such a result: 1) there is a species difference in hydrogen disposal route where sheep tend to dispose of hydrogen through an increased production of propionate while cattle form molecular hydrogen, or 2) the microbial community under development in young ruminants lacks the metabolic flexibility to shift to the propionate pathway. The first scenario seems to be the less likely because methane inhibition using chloroform in adult cattle [9] has shown to increase the propionate proportion in the rumen significantly, with a similar decrease in acetate to propionate ratio (A/P) as observed in our experiments with sheep (Milestone report 2). In the current experiment, there was no change in the A/P ratio (**Error! Reference source not found.**).

Table 13 Proportion of propionate [%] of the calves during the course of the experiment

Time	Co	T5	T10	sed	P value
Week 3	26.8	21.2	24.0	4.48	0.497
Week 5	22.0	20.1	23.9	4.48	0.707
Week 7	24.6	26.4	22.5	5.26	0.768
Week 10	19.3	18.8	21.7	3.79	0.730
Week 12	19.0	24.1	19.8	3.08	0.257
Week 15	17.4	14.9	18.5	1.69	0.150
Week 19	20.3	20.4	21.3	2.18	0.874
Week 23	31.1	49.9	41.0	9.83	0.216
sed	11.86	14.19	13.11		
P value	<0.001	<0.001	0.040		

sed, standard error of the differences;
grey shaded cells represent times when methane inhibitors were applied

Table 14 Acetate to propionate ratio of the calves during the course of the experiment

Time	Co	T5	T10	sed	P value
Week 3	2.3	2.9	2.3	0.34	0.234
Week 5	2.8	2.6	2.2	0.47	0.592
Week 7	2.5	2.1	2.5	0.67	0.801
Week 10	3.6	3.5	3.2	0.99	0.999
Week 12	3.3	2.4	3.2	0.65	0.589
Week 15	3.7	4.6	3.7	0.40	0.117
Week 19	3.0	3.1	3.0	0.37	0.921
Week 23	4.6	4.1	4.5	0.37	0.393
sed	0.54	0.44	0.67		
P value	0.008	<0.001	0.057		

sed, standard error of the differences;
grey shaded cells represent times when methane inhibitors were applied

Based on these results, the most likely explanation for the differences in hydrogen disposal between adult sheep and calves is the inability of the developing microflora in a young rumen to shift to low hydrogen producing pathways. Such a lack of metabolic flexibility of the very young ruminant could explain the lack of a response in methane emissions after the treatments stopped, and as it indicates that the most sensitive time to change the rumen community is not during the first weeks of life but later possibly after weaning when a fully functional microbial community in the rumen is developing.

Archaeal community

Archaea were dominated by Methanobrevibacter species during the whole experimental period, and during the times when inhibitors were applied, no significant differences in the proportion Methanobrevibacter was observed (**Error! Reference source not found.**a). Differences observed for Methanosphaera were not consistent with the inhibitor treatments (**Error! Reference source not found.**b). The composition of the methanogen community in this experiment was unaffected by the inhibitors used. This is a very unique finding since different methanogen groups appear to be affected to different degrees by inhibitors like halogenated methane analogues (Knight et al., 2011, Abecia et al., 2013).

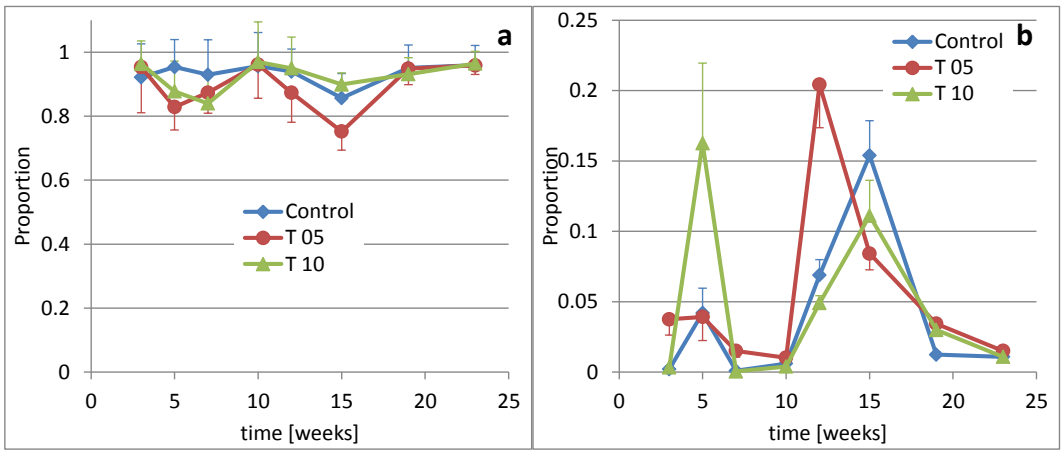

Figure 11 Proportion Methanobrevibacter (a) and Methanosphrea (b) in calves where methane emissions were inhibited over a period of 5 or 10 weeks

Bacterial community

Analysis of the bacterial community included assessment of the effect of treatment and time. Significant differences the bacterial community were observed (P = 0.002) before weaning at week 10 and after weaning (**Error! Reference source not found.**). These differences however occurred in both the control and the treatment group and reflect the change in diet from the starter meal to a roughage diet and this community change is also illustrated by their increased proportions of the major fibrolytic bacteria species shown in **Error! Reference source not found.**. However, even during period 1, the treatment period when inhibitors were fed to the animals, there was no difference in bacterial community composition between the treated and the control animals. None of the bacterial species differed significantly between treatment groups at any stage evaluated.

The lack of effect of methane inhibition on bacterial species is an unexpected result, since methane inhibition increased the hydrogen concentration in the rumen (Milestone report 3) but this did not lead to a change microbial community composition or a change in ruminal fermentation pathways. These results are in contradiction to the general concept of hydrogen disposal in the rumen (Latham and Wolin, 1977, Janssen, 2010) where in response to an increased hydrogen pressure, the hydrogen consuming propionate production in the rumen is increases which this is accompanied by a change in the bacterial composition *in vitro* (Agarwal et al., 2008, Goel et al., 2009). We are currently not aware of any studies on the effect of methane inhibition on the bacterial community composition *in vivo*. To date, most *in vivo* studies have only focused on the effect of methane inhibition on the methanogen community.

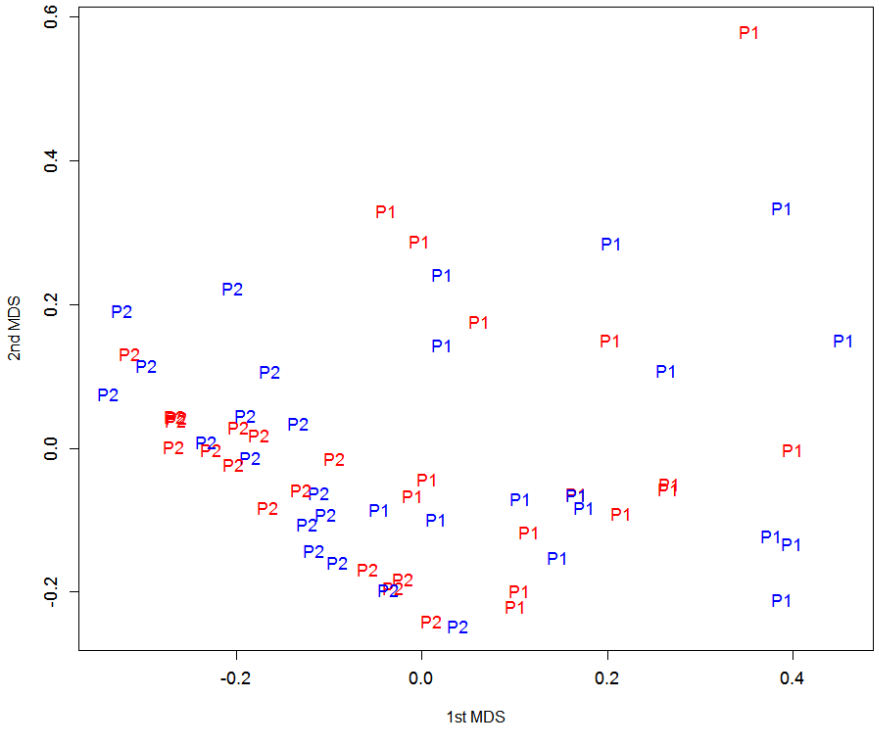

Figure 12 Multi-dimensional scaling (MDS) based on all 99 bacterial species and 62 observations during the inhibitor period (P1) and the recovery period (P2) for treated (T10, blue) and control (red) animals

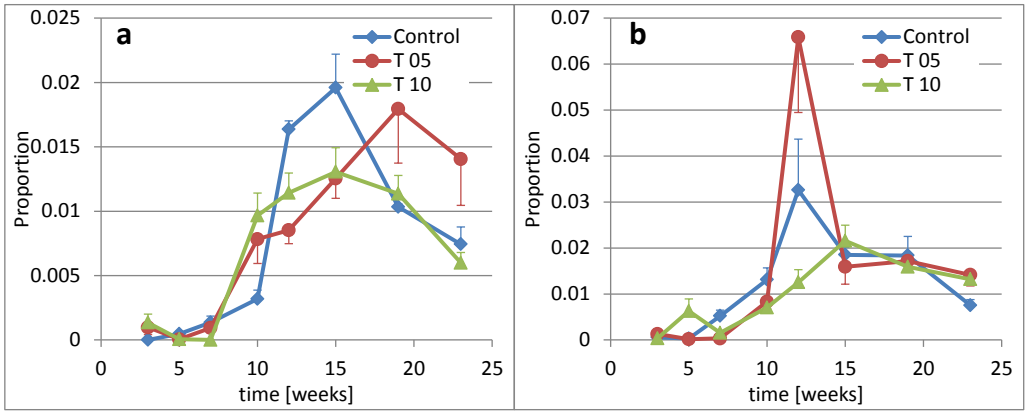

Figure 13 Proportion of Ruminococcus flavefaciens (a) and Fibrobacter succinogenes (b) in control and treated calves where methane emissions were inhibited over a period of 5 or 10 weeks.

No protozoa were observed until week 20 with the exception of one animal where some protozoa were detected in week 15. At week 20, the protozoal composition in all the animals was dominated by Entodinium species (92%) but rapidly developed to a more complex community at week 23 consisting of Entodinium, Epidinium and Eudiplodinium accounting for 33, 55 and11% in the total protozoal population, respectively.

However, this is a preliminary study with very limited number of animals and the results will have to be confirmed in the larger study which will be carried out this financial year. In addition we have suggested a separate study with young sheep using the same diet and inhibitor combination as used in the current programme to test our hypothesis regarding the lack of metabolic flexibility in a very young rumen.
This study is the first description of hydrogen disposal as molecular hydrogen in animals without a change in fermentation end products and shows the flexibility of the rumen microbial ecosystem to function normally when terminal hydrogen disposal is perturbed. Because of the unique results generated in this pilot trial, validation of this trial with larger animal numbers is recommended.

CONCLUSION

The primary hypothesis of the experiment that methane inhibition early in life has a lasting effect on the methane production following removal of the inhibitors could not be confirmed. We have however demonstrated that methane inhibition does not impair DMI or ADG of calves. To our knowledge this is the first study where methane inhibition in ruminants had no effect on either the SCFA composition or bacterial or archaeal community.
Surprisingly, the hydrogen disposal of calves is very different from that of sheep and it is not yet clear whether this difference is due to maturity of the rumen microbial community or an inherent difference between sheep and cattle.

Objective 2, Milestone 5 & 10: Effect of methane inhibitors in early life on methane emissions, growth performance and rumen function

BACKGROUND

The rumen microbial community is composed of bacteria, protozoa and fungi. These organisms break down ingested feed, including cell walls, which are not accessible to the ruminants own digestive enzymes. This process occurs under the exclusion of oxygen and results in the production of acetate, propionate and butyrate, which are the principle form of energy for ruminants. As a by-product of the fermentation in the rumen, hydrogen and carbon dioxide are formed and a specific group of microorganisms called methanogens convert these two gases to methane and water (Janssen, 2010).

Methane is a greenhouse gas that has a 25 times higher global warming potential than carbon dioxide. In New Zealand, roughly one third of the greenhouse gas emissions originate from the enteric fermentation of ruminants. There is a large research effort to decrease or prevent methane emissions from ruminants by means of genetic selection of ruminants, low methane feeds, immunisation against methanogens and chemical inhibitors of methanogens.

Other than dietary interventions or chemical inhibitors, which have to be applied on a daily basis, a permanent change in the rumen microbial community composition would be more cost effective and applicable to the New Zealand pastoral production systems. From previous work on rumen function it is known that there are large individual differences in the microbial community of ruminants of the same species, but it is virtually impossible to change the makeup of the community in an adult ruminant (Klieve et al., 2012). However, it has been shown that early life interventions, when the rumen community is developing, can lead to persistent changes in the microbial community composition in goats (Abecia et al., 2013, Abecia et al., 2014). Such a short term early life intervention could lead to a positive a low methane rumen by leaving an imprint in the microbial community. All these changes in rumen function however, must not decrease animal productivity. The aim of this study was to determine whether early life methane inhibition (Milestone 5) leads to a lower methane rumen in the growing and adult animals (Milestone 10).

MATERIALS AND METHODS

Experimental design

Two groups of calves (n = 12) were reared either on a control diet or the control diet with methane inhibitors added. The treatment lasted for 13 weeks (inhibitor period) followed by a 10 month period where both groups were kept as one mob on pasture.

Animal handling

The calves (Friesian x Jersey) were purchased from one commercial farm at 4 days of age. While the animals were still at the farm, they were given to colostrum only. A two day supply of colostrum was collected with the calves when they were transferred to the Animal Facility at AgResearch Grasslands. On arrival the calves were weighed, allocated to treatment group balanced for live weight and housed indoors in individual pens (1.5 x 3 m).

After feeding colostrum during the first 4 weeks of life, the calves were offered 2 L (125 g/kg dry matter) of calf milk replacer (Milligans, Oamaru, NZ) at 0800 h and 1600 h, and a starter meal was offered (Denver Stock Feeds, Palmerston North; Table 15). From week 4, the calves were transitioned to a once per day (0800h) 2 L milk feeding regime and a hay-based general purpose diet (GP; Table 15Table 8) was offered in addition to the starter meal. The ingredients used in the starter meal and the GP diet are given in Table 1 and the chemical composition is shown in Table 16. Intake of milk replacer, starter meal and GP was determined on a daily basis. Water was available *ad libitum*. At 10 weeks of age the calves were weaned from milk over 10 days by a stepwise reduction in milk volume. After weaning from milk replacer, the calves were group housed in a pen indoors, per treatment, for 4 weeks and transitioned to the GP diet by gradually reducing the starter meal. At week 14, the calves were transferred to a paddock with good quality grass and the amount of GP offered daily reduced in a stepwise fashion over a 3 week period.

The inhibitors used in this study were 9,10-anthraquinone (AQ) and chloroform (CF). CF was complexed with cyclodextrin to obtain a white odourless paste that prevents CF from evaporating. Concentrations of AQ and CF were 500 and 50 mg/kg for the starter meal and GP diet, respectively. Both AQ and CF were first mixed into 1/5 of the amount of starter meal required using a food processor and then mixed into the total batch amount using a commercial concrete mixer. For the GP diet, the inhibitors were premixed into the soy bean meal. Batches of GP were prepared using a mixing wagon approximately every 4 days and stored in a cold room (4°C) until used. Intake of starter meal and GP therefore determined total inhibitor intake. The inhibitors were included in the starter meal and GP diet until week 14, when the animals went onto pasture in one mob. Calf live weight was determined weekly during the first 12 weeks of life and fortnightly until 6 months of age, and then monthly thereafter.

Methane emissions were measured at 2, 4, 6, 8, 10, 14, 24, 49 and 62 weeks of age. Measurements were carried out in large cattle respiration chambers for 24 h, and the air flow was set at 600 L/min to account for the lower methane production rates of young animals compared with mature cattle. For the last two methane measurements, the airflow was increased to 1000 and 1500 L/min, respectively, to account for the increased animal size. Solid feed (starter meal and GP) was offered in the morning in the chambers and water was available ad libitum. During the initial measurements when the animals received milk replacer, it was offered to the calves in the morning and in the afternoon as above. During the last three methane measurements, pasture was cut daily at Aorangi Farm, transported to the Animal Facility at Grasslands and offered *ad libitum*.

After the calves were removed from the chambers, rumen fluid samples were collected via stomach tube to determine rumen fermentation parameters and microbial communities. The samples were subsampled for short chain fatty acid (SCFA) and ammonia (NH_4) analysis (1.8 ml) and DNA extraction (0.9 ml). DNA samples were snap-frozen and stored at -20°C. Samples for SCFA analysis were centrifuged (20,000 g, 10 min, 4 °C), and an aliquot of 0.9 ml of the supernatant collected into 0.1 ml of internal standard (19.8 mM ethylbutyrate in 20% v/v phosphoric acid) and stored at -20 °C till SCFA and NH_4 analysis. SCFA samples were thawed and centrifuged (20,000 g, 10 min, 4 °C) and 800 µl of the supernatant were collected into a crimp cap glass vial. SCFA were analysed in a HP 6890 gas chromatograph as

described by (Attwood et al., 1998). The remaining supernatant was collected for ammonia analysis according to the method of (Weatherburn, 1967).

Table 15 Ingredients of the starter meal and the general purpose (GP) diet

Starter Meal	g/kg	**GP diet**	g/kg
Maize	108	Hay	500
Barley	432	Barley	290
Peas	173	Soybean meal	100
Soybean meal	205	Molasses	100
Dried molasses	54	Di-calcium-phosphate	5.5
Sodium bicarbonate	20	Salt	3.0
Salt	5.0	Mineral/vitamin mix	1.5
Calf pre-mix	1.0		
Bovatec®	0.6		
Rumasweet palatant	0.2		

Table 16 Chemical composition (g/kg) of the calf milk replacer (CMR), starter meal and the general purpose (GP) diet

Diet	CMR	Starter Meal	GP diet
Dry matter	970	887	828
Ash	62	59	56
Protein	227	208	133
Fat	206	17	11
Neutral detergent fibre		155	438
Sugars	495*		51

[1] Manufacturers data, * Lactose

Statistical Analysis

Data was analysed using the ANOVA procedure (Genstat V13) comparing the effects of the treatments during the two periods of the experiment. P values < 0.05 were declared significant.

RESULTS AND DISCUSSION

Dry matter intake and gas emissions

During the treatment period, the inhibition of methane emissions was more than 80% and large emissions of hydrogen were observed (Table 17, Figure 14). This is in contrast to the hydrogen emissions in sheep fed the same concentration of inhibitors (Milestone 3 report), but in agreement with the high hydrogen emissions observed in dairy cows fed inhibitors (Milestone 9 report). In the literature there is ample evidence that methane can be reduced in sheep and cattle when providing methane inhibitors (Knight et al., 2011, Martínez-Fernández et al., 2014), however in most studies hydrogen is not measured and there are even less studies where methane has been inhabited in young animals. Methane emissions have been reduced in goat kids (Abecia et al., 2013) and in this experiment the effect was persistent into the post weaning period when no inhibitors were fed anymore. This is in direct contrast to our results, where neither methane nor hydrogen emissions were affected by the early life treatment of the calves with the inhibitors. However, the post-weaning effect in the goats was only observed when the mothers were also treated with inhibitors pre-partum (Abecia et al., 2013). Unfortunately, no hydrogen emissions were reported for the goat kids. A microbial analysis of the rumen samples will be carried out to determine whether the methane inhibition led to a microbial imprint.

The high hydrogen emissions in our experiment did not appear to have any effect on dry matter intake (Figure 15). There is evidence that hydrogen decreases fibre degradation, but this has been demonstrated only in pure culture experiments *in vitro*. There was also no effect of the hydrogen on the live-weight of the calves over time (Figure 16). Using bromochloromethane as an methane inhibitor in sheep did not change DMI, weight gain or fibre digestibility (Sawyer et al., 1974) and recent experiments with 3-n1trooxypropanol have also shown little effect of methane inhibition on production parameters in beef and dairy cattle (Haisan et al., 2014, Reynolds et al., 2014). During the post weaning period when the animals were fed fresh pasture, DMI, methane and hydrogen emission were similar between the control and previously inhibited groups.

Table 17 Dry matter intake and gas emissions of the two groups of calves during the treatment period where inhibitors were offered and during the recovery period.

	Treatment period		Recovery period			
Parameter	Control	Inhibitor	Control	Inhibitor	SED	P value
DMI [kg/d]	1.23	1.10	5.72	5.61	0.32	<0.001
CH4 [g/d]	24.8	3.7	117.8	115.4	5.55	<0.001
H2 [g/d]	0.14	2.87	0.08	0.14	0.30	<0.001
CH4 [g/kg DM]	18.7	3.6	21.7	21.2	1.34	<0.001

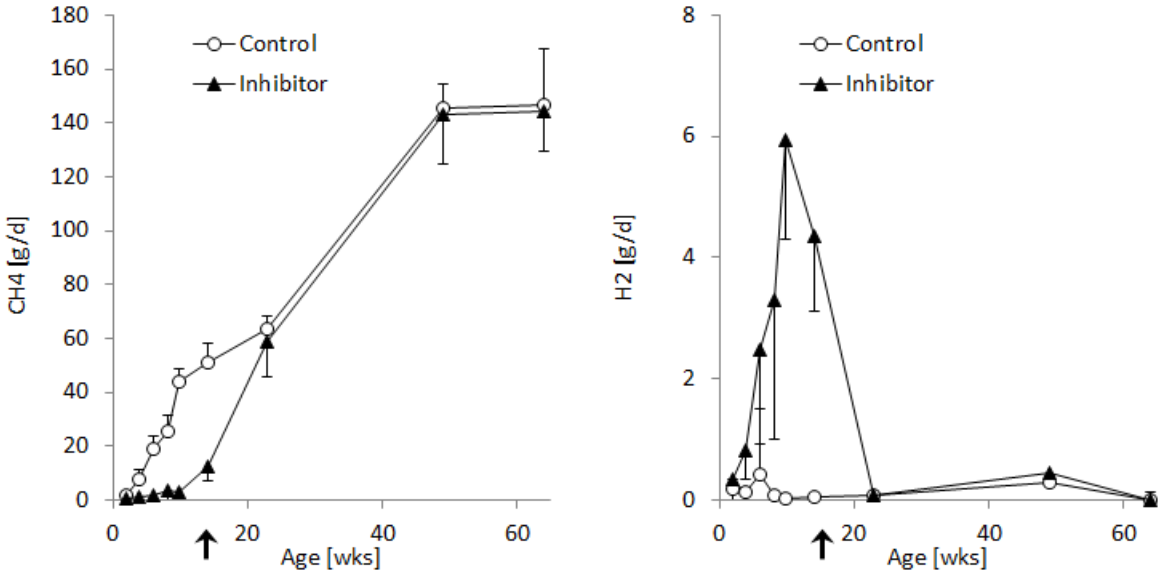

Figure 14 Methane (CH4) and hydrogen (H2) (average and standard deviation) emissions of calves offered methane inhibitors during treatment and recovery period. (The arrow indicates the end of the inhibitor period)

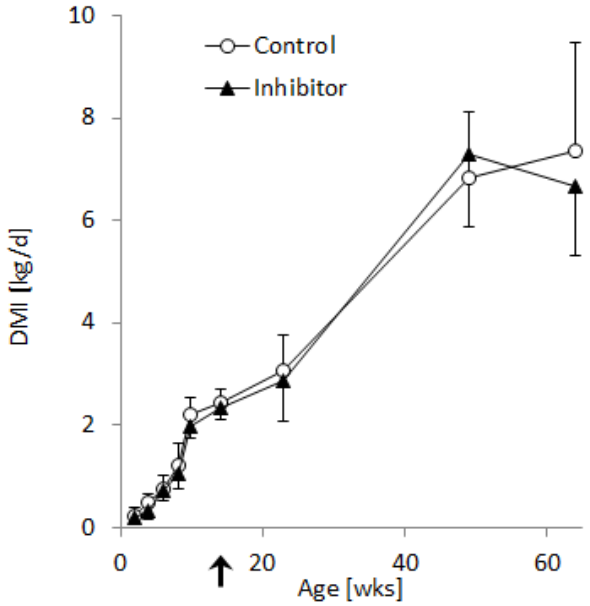

Figure 15 Dry matter intake (average and standard deviation) of calves from calves offered methane inhibitors during treatment and recovery period. (The arrow indicates the end of the inhibitor period)

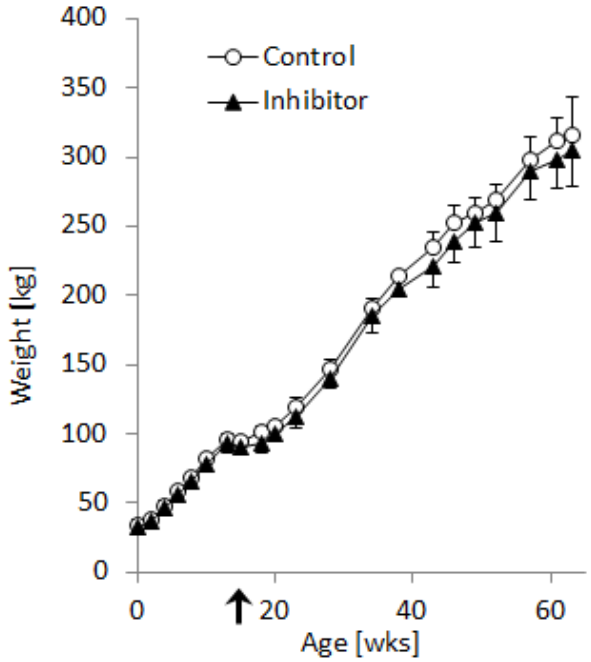

Figure 16 Average weight (average and standard deviation) of calves from calves offered methane inhibitors during treatment and recovery period. (The arrow indicates the end of the inhibitor period)

There was no significant difference in the concentration of SCFA between the two groups during both measurement periods (Table 4). In recovery period, less SCFA were observed compared to the treatment period (Table 18). During the treatment period, the proportion of acetate was lower for the inhibitor group, while propionate and butyrate concentrations were increased. The minor fatty acids valerate and caproate were also increased in the inhibitor group compared to the control group. The protein degradation product isobutyrate was similar between the two groups while the protein degradation product isovalerate proportion was increased in the inhibitor group. All short chain fatty acid proportions were similar during the recovery period, which is in accordance with the lack of an effect in the methane emissions.

Table 18 Short chain fatty acid (SCFA) concentration and composition in the rumen of the two groups of calves during the treatment period where inhibitors were offered and during the recovery period.

	Treatment period		Recovery period			
Parameter	Control	Inhibitor	Control	Inhibitor	sed	P value
SCFA [mM]	81.3	77.7	69.0	68.9	3.68	<0.001
Acetate [%]	59.6	48.0	67.1	66.4	1.21	<0.001
Propionate [%]	22.7	30.2	17.5	18.0	1.14	<0.001
Butyrate [%]	11.5	12.9	9.3	9.5	1.04	<0.001
Valerate [%]	1.96	3.55	1.11	1.18	0.21	<0.001
Caproate [%]	0.74	1.17	0.25	0.29	0.14	<0.001
Isobutyrate [%]	0.92	0.90	1.39	1.38	0.07	<0.001
Isovalerate [%]	1.16	1.79	1.68	1.65	0.24	0.011

CONCLUSION

Based on the current data, there is no evidence that methane inhibition in early life has an effect on methane emissions or rumen metabolites concentration or composition later in life. This is despite the micro flora, in the treated calves, being exposed to high hydrogen conditions for the first 13 weeks of the calf's life. However, to test for an effect of the early life intervention a real challenge might be needed. The inhibitor group has been exposed to high hydrogen conditions which are experienced in dairy cattle when supplements or high energy feeds are offered during lactation.

We therefore suggest challenging these calves with a concentrate or fodder beet to determine whether the fermentation pathways and methane emissions differ under those high hydrogen conditions.

Conclusions and Recommendations

In contrast to the results from pure culture experiments with fibre degrading bacteria, the results from this research programme indicated that hydrogen gas, as an alternative form of hydrogen disposal when methane emissions were inhibited, did not negatively affect rumen fermentation or total tract fibre digestibility, which is one of the most important functions of the rumen. The negative effects of the inhibitors on dry matter intake in adult animals were most likely attributed to direct effects of the inhibitors on the animal and not due to molecular hydrogen accumulation in the rumen. The changes in rumen fermentation pathways were different for sheep and cattle, which means that the effectiveness of new inhibitors might have to be evaluated in both species before its use on farm. Results of this programme indicate that no alternative hydrogen disposal treatments will be required when methanogen inhibitors are used.

The hypothesis that a short term intervention in early life can lead to a microbial/metabolic imprint in the rumen with lasting effects later in life could not be verified. Methane emissions in calves were reduced by 90% over a 10 week period when feeding methanogen inhibitors, but methane emission and fermentation parameters reverted to similar levels as in the control when the inhibitor treatments were removed from the diet. However, the efficacy of early life treatments should be tested under conditions similar to those generated in early life, a conclusion reached from an aligned workshop conducted as part of the GRA Senior Scientist visit by David Yanez-Ruiz, an international leader in the area of early-life interventions in ruminants.

As outlined in the proposal for this contract, we have kept the dairy calves from objective 2 on farm and we recommend to test for metabolic imprinting of the rumen when these animals mature, by feeding these animals with a highly fermentable concentrate diet during lactation. This dietary treatment will create a high molecular hydrogen environment in the rumen similar to their early life methanogen inhibitor treatments. This will generate a rumen environment suitable to test the hypothesis that methane emissions from the treated animals will be lower since their microbiome has been primed to these conditions early in life.

REFERENCES

A.O.A.C. 1990. Official methods of analysis. Association of Official Analytical Chemists, Washington, DC.

Abecia, L., I. Martin-Garcia, G. Martinez, C. J. Newbold, and D. R. Yanez-Ruiz. 2013. Nutritional intervention in early life to manipulate rumen microbial colonization and methane output by kid goats post-weaning. Journal of Animal Science 91:4832-4840.

Abecia, L., E. Ramos-Morales, G. Martínez-Fernandez, A. Arco, A. I. Martín-García, C. J. Newbold, and D. R. Yáñez-Ruiz. 2014. Feeding management in early life influences microbial colonisation and fermentation in the rumen of newborn goat kids. Animal Production Science 54(9):1449-1454.

Abecia, L., P. G. Toral, A. I. Martín-García, G. Martínez, N. W. Tomkins, E. Molina-Alcaide, C. J. Newbold, and D. R. Yáñez-Ruiz. 2012. Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. Journal of Dairy Science 95(4):2027-2036.

Agarwal, N., D. N. Kamra, P. N. Chatterjee, R. Kumar, and L. C. Chaudhary. 2008. In vitro methanogenesis, microbial profile and fermentation of green forages with buffalo rumen liquor as influenced by 2-bromoethanesulphonic acid. Asian-Australasian Journal of Animal Sciences 21(6):818-823.

Aschenbach, J. R., N. B. Kristensen, S. S. Donkin, H. M. Hammon, and G. B. Penner. 2010. Gluconeogenesis in dairy cows: The secret of making sweet milk from sour dough. International Union of Biochemistry and Molecular Biology Life 62(12):869-877.

Attwood, G. T., A. V. Klieve, D. Ouwerkerk, and B. K. C. Patel. 1998. Ammonia-hyperproducing bacteria from New Zealand ruminants. Applied and Environmental Microbiology 64(5):1796-1804.

Belanche, A., J. Balcells, G. de la Fuente, D. R. Yañez-Ruíz, M. Fondevila, and L. Calleja. 2010. Description of development of rumen ecosystem by PCR assay in milk-fed, weaned and finished lambs in an intensive fattening system. Journal of Animal Physiology and Animal Nutrition 94(5):648-658.

Berends, H., C. G. van Reenen, N. Stockhofe-Zurwieden, and W. J. J. Gerrits. 2012. Effects of early rumen development and solid feed composition on growth performance and abomasal health in veal calves. Journal of Dairy Science 95(6):3190-3199.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Pẽa, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7(5):335-336.

Chong, L., Z. Zhuping, G. Tongjun, L. Yongming, and D. Hongmin. 2013. Changes in methane emission, rumen fermentation, and methanogenic community in response to silage and dry cornstalk diets. Journal of Basic Microbiology 54(6):521-530.

Clark, H., F. Kelliher, and C. Pinares-Patino. 2011. Reducing CH4 emissions from grazing ruminants in New Zealand: Challenges and opportunities. Asian-Australasian Journal of Animal Sciences 24(2):295-302.

Finlay, B. J., G. Esteban, K. J. Clarke, A. G. Williams, T. M. Embley, and R. P. Hirt. 1994. Some rumen ciliates have endosymbiotic methanogens. FEMS Microbiology Letters 117(2):157-162.

Goel, G., H. P. S. Makkar, and K. Becker. 2009. Inhibition of methanogens by bromochloromethane: Effects on microbial communities and rumen fermentation using batch and continuous fermentations. British Journal of Nutrition 101(10):1484-1492.

Gomez-Alarcon, R. A., C. O'Dowd, J. A. Leedle, and M. P. Bryant. 1982. 1,4-Naphthoquinone and other nutrient requirements of Succinivibrio dextrinosolvens. Applied and Environmental Microbiology 44(2):346-350.

Haisan, J., Y. Sun, L. L. Guan, K. A. Beauchemin, A. Iwaasa, S. Duval, D. R. Barreda, and M. Oba. 2014. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation. Journal of Dairy Science 97(5):3110-3119.

Hammond, K. J., S. O. Hoskin, J. L. Burke, G. C. Waghorn, J. P. Koolaard, and S. Muetzel. 2011. Effects of feeding fresh white clover (Trifolium repens) or perennial ryegrass (Lolium perenne) on enteric methane emissions from sheep. Animal Feed Science and Technology 166-167:398-404.

Itabashi, H. and M. kandatsu. 1978. Formation of methylamine by rumen microorganisms. Japanese Journal of Zootechnical Science 49(2):110-118.

Janssen, P. H. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Animal Feed Science and Technology 160(1-2):1-22.

Johnson, D. E. 1974. Adaptational Responses in Nitrogen and Energy Balance of Lambs Fed a Methane Inhibitor. Journal of Animal Science 38(1):154-157.

Johnson, K. A. and D. E. Johnson. 1995. Methane emissions from cattle. Journal of Animal Science 73(8):2483-2492.

Kittelmann, S., H. Seedorf, W. A. Walters, J. C. Clemente, R. Knight, J. I. Gordon, and P. H. Janssen. 2013. Simultaneous Amplicon Sequencing to Explore Co-Occurrence Patterns of Bacterial, Archaeal and Eukaryotic Microorganisms in Rumen Microbial Communities. PLoS ONE 8(2):e47879.

Klieve, A. V., S. R. McLennan, and D. Ouwerkerk. 2012. Persistence of orally administered Megasphaera elsdenii and Ruminococcus bromii in the rumen of beef cattle fed a high grain (barley) diet. Animal Production Science 52(5):297-304.

Knight, T., R. S. Ronimus, D. Dey, C. Tootill, G. Naylor, P. Evans, G. Molano, A. Smith, M. Tavendale, C. S. Pinares-Patiño, and H. Clark. 2011. Chloroform decreases rumen methanogenesis and methanogen populations without altering rumen function in cattle. Animal Feed Science and Technology 166-167:101-112.

Kung Jr, L., K. A. Smith, A. M. Smagala, K. M. Endres, C. A. Bessett, N. K. Ranjit, and J. Yaissle. 2003. Effects of 9,10 anthraquinone on ruminal fermentation, total-tract digestion, and blood metabolite concentrations in sheep. Journal of Animal Science 81(1):323-328.

Latham, M. J. and M. J. Wolin. 1977. Fermentation of cellulose by Ruminococcus flavefaciens in the presence and absence of Methanobacterium ruminantium. Applied and Environmental Microbiology 34(3):297-301.

Marounek, M., K. Fliegrova, and S. Bartos. 1989. Metabolism and some characteristics of ruminal strains of Megasphaera elsdenii. Applied and Environmental Microbiology 55(6):1570-1573.

Martínez-Fernández, G., L. Abecia, A. Arco, G. Cantalapiedra-Hijar, A. I. Martín-García, E. Molina-Alcaide, M. Kindermann, S. Duval, and D. R. Yáñez-Ruiz. 2014. Effects of ethyl-3-nitrooxy propionate and 3-nitrooxypropanol on ruminal fermentation, microbial abundance, and methane emissions in sheep. Journal of Dairy Science 97(6):3790-3799.

Mathers, J. C. and E. L. Miller. 1982. Some Effects of Chloral Hydrate on Rumen Fermentation and Digestion in Sheep. Journal of Agricultural Science 99(1):215-224.

McAllister, T. A. and C. J. Newbold. 2008. Redirecting rumen fermentation to reduce methanogenesis. Australian Journal of Experimental Agriculture 48(1-2):7-13.

McCrabb, G. J., K. T. Berger, T. Magner, C. May, and R. A. Hunter. 1997. Inhibiting methane production in Brahman cattle by dietary supplementation with a novel compound and the effects on growth. Australian Journal of Agriculture Research 48(3):323-329.

Min, B. R., W. E. Pinchak, R. C. Anderson, and M. E. Hume. 2006. In vitro bacterial growth and in vivo ruminal microbiota populations associated with bloat in steers grazing wheat forage. Journal of Animal Science 84(10):2873-2882.

Mitsumori, M., T. Shinkai, A. Takenaka, O. Enishi, K. Higuchi, Y. Kobayashi, I. Nonaka, N. Asanuma, S. E. Denman, and C. S. McSweeney. 2012. Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. British Journal of Nutrition 108(3):482-491.

Neill, A. R., D. W. Grime, and R. M. Dawson. 1978. Conversion of choline methyl groups through trimethylamine into methane in the rumen. Biochemical Journal. 170(3):529-535.

Pinares-Patiño, C. S., P. Lovejoy, C. S. Hunt, R. Martin, G. Molano, G. C. Waghorn, and H. Clark. 2008. Brief communication: A versatile sheep respiration chamber system for measurement of methane emissions Proceedings of the New Zealand Society of Animal Production 68:29-30.

Popova, M., D. P. Morgavi, and C. Martin. 2013. Methanogens and methanogenesis in the rumens and ceca of lambs fed two different high-grain-content diets. Applied and Environmental Microbiology 79(6):1777-1786.

Poulsen, M., C. Schwab, B. Borg Jensen, R. M. Engberg, A. Spang, N. Canibe, O. Højberg, G. Milinovich, L. Fragner, C. Schleper, W. Weckwerth, P. Lund, A. Schramm, and T. Urich. 2013. Methylotrophic methanogenic Thermoplasmata implicated in reduced methane emissions from bovine rumen. Nature Communications 4(1428).

Reynolds, C. K., D. J. Humphries, P. Kirton, M. Kindermann, S. Duval, and W. Steinberg. 2014. Effects of 3-nitrooxypropanol on methane emission, digestion, and energy and nitrogen balance of lactating dairy cows. Journal of Dairy Science 97(6):3777-3789.

Rius, A. G., S. Kittelmann, K. A. Macdonald, G. C. Waghorn, P. H. Janssen, and E. Sikkema. 2012. Nitrogen metabolism and rumen microbial enumeration in lactating cows with divergent residual feed intake fed high-digestibility pasture. Journal of Dairy Science 95(9):5024-5034.

Robertson, J. B. and P. N. Van Soest. 1981. The detergent system of analysis and its application to human foods. Page 123 in The analysis of dietary fibre in foods. W. P. T. James and T. O, ed. Marcel Dekker, New York, NY.

Sawyer, M. S., W. H. Hoover, and C. J. Sniffen. 1974. Effects of a Ruminal Methane Inhibitor on Growth and Energy Metabolism in the Ovine. Journal of Animal Science 38(4):908-914.

Schulman, M. D. and D. Valentino. 1976. Factors Influencing Rumen Fermentation: Effect of Hydrogen on Formation of Propionate. Journal of Dairy Science 59(8):1444-1451.

Shinkai, T., O. Enishi, M. Mitsumori, K. Higuchi, Y. Kobayashi, A. Takenaka, K. Nagashima, and M. Mochizuki. 2012. Mitigation of methane production from cattle by feeding cashew nut shell liquid. Journal of Dairy Science 95(9):5308-5316.

Smolenski, W. J. and J. A. Robinson. 1988. In situ rumen hydrogen concentrations in steers fed eight times daily, measured using a mercury reduction detector. FEMS Microbiology Letters 53(2):95-100.

Soberon, F., E. Raffrenato, R. W. Everett, and M. E. Van Amburgh. 2012. Preweaning milk replacer intake and effects on long-term productivity of dairy calves. Journal of Dairy Science 95(2):783-793.

Sun, X. Z., S. O. Hoskin, G. G. Zhang, G. Molano, S. Muetzel, C. S. Pinares-Patiño, H. Clark, and D. Pacheco. 2012a. Sheep fed forage chicory (Cichorium intybus) or perennial ryegrass (Lolium perenne) have similar methane emissions. Animal Feed Science and Technology 172(3-4):217-225.

Sun, X. Z., G. C. Waghorn, S. O. Hoskin, S. J. Harrison, S. Muetzel, and D. Pacheco. 2012b. Methane emissions from sheep fed fresh brassicas (Brassica spp.) compared to perennial ryegrass (Lolium perenne). Animal Feed Science and Technology 176(1-4):107-116.

Tajima, K., R. I. Aminov, T. Nagamine, H. Matsui, M. Nakamura, and Y. Benno. 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Applied and Environmental Microbiology 67(6):2766-2774.

Teunissen, M. J., E. P. W. Kets, H. J. M. Op den Camp, J. H. J. Huis in't Veld, and G. D. Vogels. 1992. Effect of coculture of anaerobic fungi isolated from ruminants and non-ruminants with methanogenic bacteria on cellulolytic and xylanolytic enzyme activities. Arch. Microbiol. 157(2):176-182.

Tokura, M., K. Ushida, K. Miyazaki, and Y. Kojima. 1997. Methanogens associated with rumen ciliates. FEMS Microbiology Ecology 22(2):137-143.

Trei, J. E., G. C. Scott, and R. C. Parish. 1972. Influence of methane inhibition on energetic efficiency of lambs. Journal of Animal Science 34(3):510-515.

Tymensen, L. D., K. A. Beauchemin, and T. A. McAllister. 2012. Structures of free-living and protozoa-associated methanogen communities in the bovine rumen differ according to comparative analysis of 16S rRNA and mcrA genes. Microbiology (United Kingdom) 158(7):1808-1817.

Ungerfeld, E. M., S. R. Rust, D. R. Boone, and Y. Liu. 2004. Effects of several inhibitors on pure cultures of ruminal methanogens. Journal of Applied Microbiology 97(3):520-526.

van Zijderveld, S. M., W. J. J. Gerrits, J. A. Apajalahti, J. R. Newbold, J. Dijkstra, R. A. Leng, and H. B. Perdok. 2010. Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. Journal of Dairy Science 93(12):5856-5866.

Weatherburn, M. W. 1967. Phenol-hypochlorite reaction for determination of ammonia. Analytical Chemistry 39(8):971-974.

Williams, A. G., S. E. Withers, and K. N. Joblin. 1994. The effect of cocultivation with hydrogen-consuming bacteria on xylanolysis byRuminococcus flavefaciens. Current Microbiology 29(3):133-138.

Wright, A. D. G., X. L. Ma, and N. E. Obispo. 2008. Methanobrevibacter phylotypes are the dominant methanogens in sheep from Venezuela. Microbial Ecology 56(2):390-394.

Wright, A. D. G., A. J. Williams, B. Winder, C. T. Christophersen, S. L. Rodgers, and K. D. Smith. 2004. Molecular diversity of rumen methanogens from sheep in western Australia. Applied and Environmental Microbiology 70(3):1263-1270.

Yanez-Ruiz, D. R., K. J. Hart, A. I. Martin-Garcia, S. Ramos, and C. J. Newbold. 2008. Diet composition at weaning affects the rumen microbial population and methane emissions by lambs. Australian Journal of Experimental Agriculture 48(1-2):186-188.