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Increasing productivity and reducing methane emissions by supplementing feed with dietary lipids

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Abstract

Methane (CH_4) is a by-product in the digestion of plant material by all cattle and sheep. Effectively it is wasted feed material and energy that could otherwise be available for animal production. It is also a major greenhouse gas (14% of Australia's emissions). Beef cattle contribute 50% of these emissions. Many lipid containing feed materials are known to reduce methane emissions, as well as increasing productivity when used as supplementary feeds. The purpose of this project is to investigate the impact of lipid containing feed additives on the suppression of methane emissions and improvements in the growth rate of steers fed a basal diet of tropical pastures.

A range of lipid supplements, Algamac 3050 (3, 5 and 7% oil inclusion in the diet), Spirulina (3%), canola oil (5 and 7% inclusion) and safflower oil (3, 5 and 7% oil inclusion in the diet), were tested *in vitro.*

Algamac 3050 at all levels of inclusion reduced methane generation whilst appearing not to affect rumen microbial population dynamics, including the methanogen population. There were no differences in dry-matter digestion or the numbers of methanogens or methanogen population structure. All levels of Algamac inclusion had similar effects, suggesting that the mechanism that lowers methane with algal oils is different to that of other oils, which directly impacts on the microbial ecosystem at higher levels of inclusion.

Canola oil at 7% inclusion appears to only transiently reduce methane generation and would not appear to be a good candidate as a feed supplement for lowering methane emissions. The effectiveness of safflower inclusion is dose related. Safflower at 7% inclusion reduces methane generation significantly and at a constant level. The inclusion at 5% was not effective at reducing methane production.

A range of lipid containing supplements were tested *in vivo* in a feeding trial using *Bos indicus* cross steers. The supplements included Algamac, sunflower oil (due to the commercial unavailability of safflower oil) and whole cotton seed. All lipid supplements were included at a rate of 50g lipid/kgDM. Supplementation increased live-weight gain over an 11 week period. The highest gain was from whole cottonseed supplementation (15.7kg) followed by Algamac supplementation (12.1kg). Supplementation of Algamac and sunflower oil reduced methane emissions on a live-weight and dry matter intake basis by around 22% and 19.4% respectively. Using these functions there was no reduction in methane emissions per head with whole cottonseed supplementation, however, when calculating methane emissions as a function of average daily gain (ADG), both whole cottonseed and Algamac supplementation had a four-fold reduction in emissions. Taking this reduction into account plus the fact that increasing live-weight would reduce the number of days to market, the overall reduction in methane emitted would be substantial. It is recommended the use of whole cottonseed and Algamac as a supplement to cattle fed a basal diet of tropical pastures due to its positive impact on live-weight gain, and the reduction in methane per kilogram of average daily gain, however there are supplement uptake issues that need to be overcome.

Executive Summary

Methane (CH_4) is a by-product in the digestion of plant material by all cattle and sheep. Effectively it is wasted feed material and energy that could otherwise be available for animal production. Methanogenesis is a microbiological mechanism that removes hydrogen (produced by the fermentation of feed) from the rumen. Methane is also a major greenhouse gas contributing heavily to global warming with a warming potential 21 times greater than carbon dioxide. Cattle and sheep contribute 53% of Australia's total methane emissions (3 million tonnes annually or the equivalent of 63 million tonnes of carbon dioxide) (NGGIC 2007) and 14% of the nation's total greenhouse gas emissions. Beef cattle alone contribute 50% of these emissions.

It has been estimated that, if three quarters of the methane generated could be channelled into animal product, instead of lost to the atmosphere, 10% of Australia's greenhouse gas emissions could be permanently eliminated. At the same time $150 million worth of production annually (calculated as beef cattle equivalence) could be generated by Queensland's primary producers alone.

Many lipid containing feed materials are known to reduce methane generation and are attractive as nutritional supplements due to the high proportion of concentrated energy and/or protein they contain. Feeding cattle for maximum gain and more efficient CH_4 conversion is important for it reduces the proportion of feed energy lost as CH_4 each day and reduces the number of days to market. Useful lipids for the reduction in methane production include coconut oil and whole crushed oilseeds (rapeseed, sunflower and linseed). Studies have shown that on average, methane suppression could be equated to an increase in capture of ingested energy of up to 27%. These positive benefits of using oil/lipid based feed additives have to be balanced with some negative impacts, particularly on plant fibre breakdown in the rumen.

This project studied a range of potential lipid containing supplements that could be used with subtropical/tropical grazing systems. The project initially focussed on the effect of lipid containing additives on methane production using *in vitro* techniques. Lipid containing supplements that showed potential by reducing methane production were then studied *in vivo* by means of a pen trial. Supplements of interest for the pen trial included whole cottonseed (which contains 20% lipids), Algamac (which is a marine algal meal that has shown potential as a methane emission reducer in overseas trials), and sunflower oil (due to the commercial unavailability of safflower oil). The use of these supplements as potential reducers in methane generation have not been previously studied in northern Australian grazing conditions.

The project aimed to optimise methane reduction in association with improving productivity.

Specific objectives were to:

- Evaluate and compare the ability of Algamac, spirulina, safflower oil and canola oil to reduce methane generation using *in vitro,* rumen based, fermentation experiments; and
- Confirm and quantify the impact of Algamac, sunflower oil and whole cotton seed on rumen methanogenesis in cattle and determine performance benefits when fed with a production ration.

A large number of *in vitro* fermentor based experiments were undertaken to determine the impacts of Algamac, safflower oil and canola oil on methane generation, methanogen populations (numbers and diversity) and important rumen digestion parameters such as volatile fatty acid (VFA) production and the extent of

plant fibre digestion. Fermentations were performed at a number of differing concentrations (3, 5 or 7%).

Algamac 3050 at all levels of inclusion reduced methane emissions whilst appearing to not affect rumen microbial population dynamics, including the methanogen population. There were no differences in dry-matter digestion or the numbers of methanogens or methanogen population structure. All levels of Algamac inclusion had similar effects, suggesting that the mechanism that lowers methane with algal oils is different to that of other oils, which directly impact on the microbial ecosystem at higher levels of inclusion. The fresh water alga, *Spirulina*, had no effect on reducing emissions.

Canola oil at 7% inclusion appears to only transiently reduce methane generation and would not appear to be a good candidate as a feed supplement for lowering methane emissions.

The effectiveness of safflower inclusion is dose related. Safflower at 7% inclusion reduces methane emissions significantly and at a constant level. The inclusion at 5% was not effective at reducing methane production.

The impact of some lipid feed supplements was then tested in steers in a pen trial. Commercial Brahman crossbred steers (32 in total), approximately 10-12 months of age, were used in a randomised complete block design with a basal diet of Rhodes Grass (*Chloris gayana*) hay and molasses. Supplement treatments were whole cottonseed, sunflower oil and Algamac, each treatment aiming to deliver 5% lipid in the diet. Live-weight and intake (total, hay and supplement) were measured daily throughout the experiment (duration 11 weeks) and rumen samples were collected at day 50 to determine the impact on methanogen numbers and diversity. At the conclusion of the feed trial which determined the impact on live-weight gain, a 5 day methane emission measurement program (2 day preliminary and 3 day emission measurement) was undertaken in methane chambers which were modified climate control rooms. Air was sampled into 10 mL Hungate tubes and analysed by gas chromatography at the School of Agriculture and Food Sciences, University of Queensland, St Lucia.

The inclusion of whole cottonseed or Algamac in the diet, increased live-weight gain in steers by 15 kg and 12 kg respectively over an 11 week period. Concomitant with the increased productivity, there was no overall change in methane emissions per head with whole cottonseed, either on a live-weight basis or by taking dry matter intake into account. There was a 22% reduction in methane emission/head when Algamac was used as a supplement. There was however a substantial decrease in methane emitted per unit of production (3851 g CH_4/Kg ADG to 913 g CH_4/kg ADG) when either whole cottonseed or Algamac was supplemented. Palatability issues arising from the use of Algamac was evident throughout this trial and supplement intakes were well below the desired level. There were also problems regarding the consistent uptake of whole cottonseed for over half the steers in this treatment group. Regardless, the use of whole cottonseed and Algamac (even at suboptimal intakes) increased the growth rates of cattle and decreased the methane produced per unit of product (meat). Unlike with many other feeding technologies (where reduction per unit product are often accompanied by increases per head) the advantages of increased production and lowered emissions per unit of production will reduce overall on farm emissions and will not require a reduction in herd numbers to offset any per head or per unit area increases in emissions (as is often the case with other strategies).

It is recommended that the use of whole cottonseed and Algamac, at a rate of 5% lipid in the diet, will increase live-weight gain in cattle and reduce methane emissions. Individual economic evaluation of on-farm profitability should be

undertaken. In terms of greenhouse emissions, the relationships between methane emission reduction per unit of growth, cost of supplement, and uptake of supplement should be incorporated in modelling on-farm emissions and in assessing where reductions or carbon savings could be made.

Table of Contents

1.0 Background

Methane is a by-product in the digestion of plant material by all cattle and sheep. Effectively it is wasted feed material and energy that could otherwise be available for animal production. Methanogenesis is a microbiological mechanism that removes hydrogen (produced by the fermentation of feed) from the rumen. Methane is also a major greenhouse gas contributing heavily to global warming with a warming potential 21 times greater than carbon dioxide. Cattle and sheep contribute 53% of Australia's total methane emissions (3 million tonnes annually or the equivalent of 63 million tonnes of carbon dioxide) and 14% of the nation's total greenhouse gas emissions. Beef cattle alone contribute 50% of these emissions.

Many lipid containing feed materials are known to reduce methane generation and are attractive as nutritional supplements due to the high proportion of concentrated energy and/or protein they contain. Studies have shown that on average, methane suppression could be equated to an increase in capture of ingested energy of up to 27%.

In a previous study we evaluated the use of coconut oil and cottonseed oil in the diet of *Bos indicus* cross heifers in northern Australia to both reduce methane emissions and increase productivity. The primary outcomes of this work were:

- A response curve approach was taken to be able to relate oil intake to live-weight gain and methane emission across the spectrum of oil concentrations from 2 to 6% incorporation in the diet.
- A substantial decrease in methane emitted per unit of production (1500 g CH_4/Kg ADG to less than 100 g CH_4/Kg ADG) was observed.
- Concomitantly, there was no overall change in methane emissions per head. This is very significant as unlike with many other feeding technologies (where reductions per unit product are often accompanied by increases per head) the advantages of increased production and lowered emissions per unit of production will not increase overall on-farm emissions meaning that to capitalise on these reductions will not require a reduction in herd numbers to offset any per head or per unit area increases in emissions.
- The inclusion of oil fortified copra and cottonseed meals increased live-weight gain in cattle by up to 20 kg without the addition of molasses and up to 30 kg with molasses added, over a 7 week period.
- Thus, not only was it possible to increase the growth rates of cattle and decrease the methane produced per unit of product (meat) but this was achieved without increasing per head methane emissions.
- Importantly, a series of fermentor experiments demonstrated that these oils reduced emissions through a direct depression of methanogen populations and the result was not simply due to a higher plane of nutrition.

Beef production systems in northern Australia are very different to cattle enterprises in the south (dairy included), the species of cattle are different (*Bos indicus* and *B. indicus* cross cattle versus *B. taurus*), the basal forage is different (subtropical C4 grasses that are known to generate higher methane emissions and that are of poor quality on a seasonal basis), seasonal availability of feed is quite different and to compensate for the poor quality of feed in the dry season these cattle often receive supplementary rations.

Approximately 50% of Australia's beef is produced from these northern production systems and these systems, due to the C4 grasses and poor quality of feed on offer

for appreciable parts of the year, produce more methane both per head and per unit product. Feed additives are a mechanism for reducing emissions (and increasing productivity) that can be available to producers in the short term but it is imperative that additives for widely differing animal production systems are developed specifically with those systems, as importing or extrapolating from other systems in unlikely to have similar outcomes, or be feasible or practical. At the same time it is important that those researchers working with feed-additive development, work in a cooperative and collaborative manner, as synergies between systems could be apparent and beneficial.

Many lipid containing feed materials are known to reduce methane generation and are attractive as nutritional supplements due to the high proportion of concentrated energy and/or protein they contain. Lipid additions to ruminal diets reduce methane emissions by several mechanisms including the biohydrogenation of unsaturated fatty acids, enhanced propionic acid production and protozoal inhibition (Johnson and Johnson 1995). Czerkawski *et al.* (1966) demonstrated that oleic acid (18:1), linoleic acid (C18:2) and linolenic acid (C18:3), when individually infused into the rumen of sheep, reduced methane generation by providing an alternative metabolic hydrogen acceptor as they become saturated. Blaxter and Czerkawski (1966) found that long and medium chain fatty acids (C10 – C14) reduced methane production. Machmuller *et al.* (1998 and 2000) investigated and compared the effects, both *in vitro* and *in vivo,* of differing feeds with high lipid contents on rumen fermentation and methane production. It was found that linseed and sunflower seed additions reduced methane release by up to 40%. Comparatively, the use of coconut oil (predominantly C12 and C14 fatty acids) completely eliminated ciliate protozoa, as well as significantly suppressing methane production both *in vitro* and *in vivo*. At supplementation levels of 3% and 6% of the total diet, coconut oil reduced emissions by 43% and 57%, respectively. In an *in vivo* study, Machmuller and Kreuzer (1999), found a reduction in methane emissions with coconut oil, supplemented at 3.5 and 7% of the diet, of 28 and 73% respectively. On average, methane suppression in these studies could be equated to an increase in capture of ingested energy of 27%. More recently, Beauchemin *et al*. (2008) demonstrated that crushed sunflower seed, canola seed and flaxseed reduced methane production by 13%, when corrected for differences in DM intake.

Also of interest is the increasing use of microalgae due to their high lipid content, and in particular their high concentration of long chain polyunsaturated lipids. An *in vitro* study by Boeckaert *et al*. (2004) demonstrated that methane production is inhibited and there is incomplete hydrogenation of C18:2 c9c12 with the supplementation of Docosahexaenoic acid (DHA) enriched microalgae.

The reduction in methane emissions can be at the expense of fibre digestibility (Broudiscou *et al*. 1990). The inclusion of 5% or greater lipid in the diet can limit fibre digestion (Orskov *et al.* 1978). Therefore the positive benefits of using lipid based feed additives will have to be balanced with the negative impacts. The proposed project was aimed at optimising methane reduction in association with improving productivity through live-weight gain. An increase in live-weight gain would also reduce the number of days to market, further reducing methane emissions in the environment.

2.0 Project Objectives

The purpose of the project was to investigate a range of lipid based products that have the potential as feed additives for *Bos indicus* cattle for their apparent capacity to reduce enteric methane production on the suppression of both methane emissions and improvements in the growth rate of steers fed C4 grasses and molasses.

Initially the effects of spirulina (a fresh water algae), canola oil, safflower oil and Algamac (a marine algal meal) were evaluated and compared with respect to reducing methane generation and methanogen species composition in *in vitro,* rumen based, fermentation experiments.

Based on the *in vitro* studies, lipids that showed potential in reducing methane emissions were analysed in an *in vivo* pen trial using *Bos indicus* cross steers. In this study the effects of lipid supplements on rumen methanogenesis and the effects on live-weight gains were investigated. Specifically the supplementation of sunflower oil, Algamac and whole cotton seed to steers fed a basal diet of medium quality Rhodes Grass (*Chloris gayana*) hay were investigated.

The major milestones for this project were:

By 15 June, 2010 provide *in vitro* data and analysis from screening a range of lipid-based products that have potential as feed additives for *Bos indicus* cattle for their apparent capacity to reduce enteric methane production.

By 31 January, 2012 provide *in vivo* data and analysis on the effects of the most promising products in reducing methane emissions and effects on live weight gains relative to lipid additive intake.

By 31 January, 2012 define the potential increases in live weight gain and decrease in methane emissions by using selected lipid based additives to basal diets common to northern Australia but which have yet to be investigated.

3.0 Methods

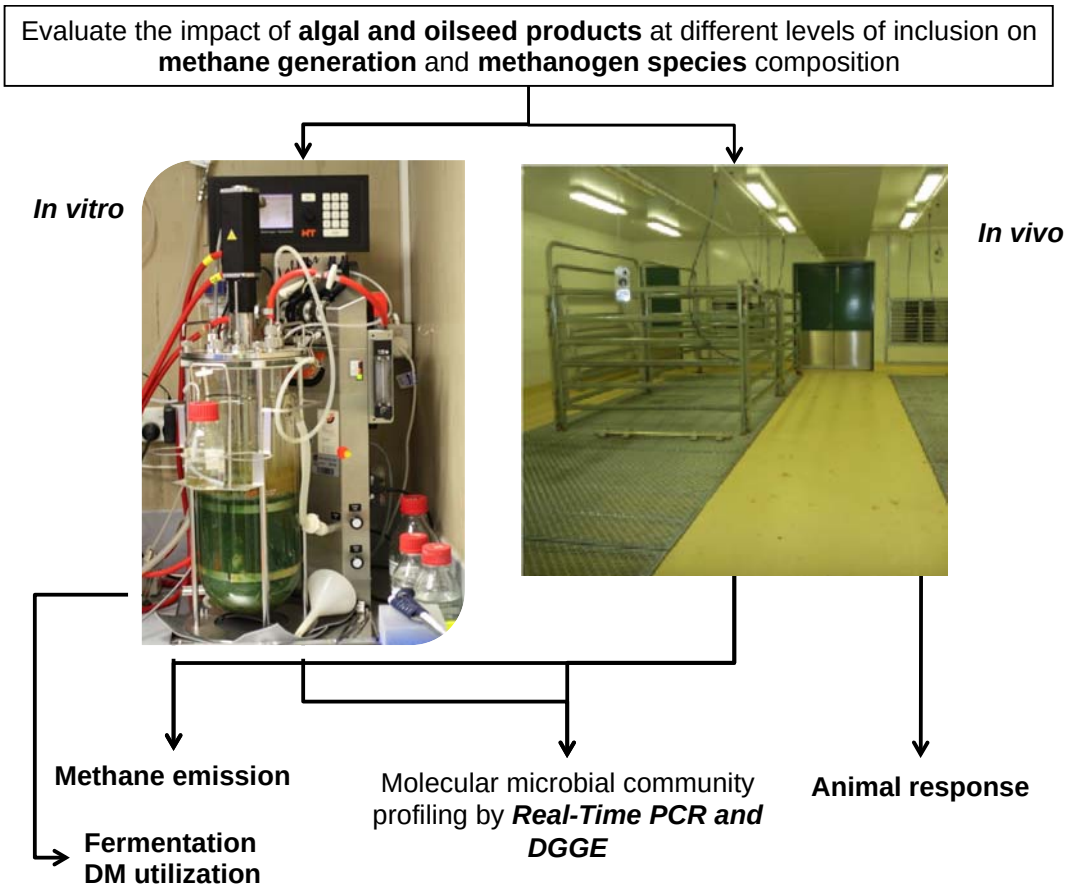


Figure 1. Overview of the methodological approach of the project.

To evaluate the ability of increasing level of lipids on diet, *in vitro* and *in vivo* approaches were utilized (Figure 1). The methane emission and molecular enumeration (real time PCR) and profiling of methanogen species using Denaturing Gradient Gel Electrophoresis (DGGE) were evaluated with both approaches. Fermentation parameters, such VFA production and dry matter utilization were measured throughout *in vitro* fermentations. The animal response of live-weight gain was evaluated with the *in vivo* study.

3.1 *In vitro* approaches of the effect of increasing dietary lipids on methanogenesis

3.1.1 Operation of the fermentor, methane analysis and fermenter fluid sampling

The methodology associated with using the fermentor is well established and the basic operation was similar to that published in relation to the production of a live inoculum for cattle grazing *Leucaena* (Klieve *et al.* 2002). A large set of starter cultures were created from a fermentation of rumen contents from a rumen cannulated steer grazing pasture supplemented with lucerne hay at the Centre for Advanced Agricultural Studies (CAAS), UQ Gatton. Fermentor liquor (50 mL aliquots) was harvested after 10 days of fermentation, combined with an equal

volume of rumen fluid/glycerol medium and stored frozen at -80^{o}C. Each fermentor run in this project was initiated with a starter culture from this set so that each run will progress in a manner similar to previous runs.

Fermentations were commenced with a 100 mL starter culture being added to 3 L of a rumen-fluid-based (RF) culture medium. At commencement of the fermentation 45 g of finely ground Mitchell Grass (*Astrebla sp*.) pasture hay was added as substrate for the fermentor. On the second day of fermentation the amount of ground hay added daily was reduced to 15 g. Each day, half of the fermentor liquid was removed and replaced with anaerobic culture medium that had been modified by the removal of most nutrients to leave a balanced salts solution. The fermentor vessel was maintained at 39^{o}C and continuously bubbled with a mixture of CO_2:H_2 (95:5 v/v) to maintain anaerobic conditions. Total fermentation time per experiment was 11 days.

Inclusion of dietary lipids. On the fifth day the diet was changed to include the supplement at either 3, 5 or 7% (w/w) of the ration (Table 1).

Table 1. The lipid sources and % inclusions tested in the *in vitro* experiments.

Lipid source	Inclusion (%)
Algamac 3050	3, 5, 7
Canola oil	5, 7
Spirulina	3
Safflower oil	5, 7

For each lipid source, two fermenter control runs (0% inclusion) and two runs for each percentage of inclusion were undertaken.

Methane production. Methane concentrations in the outflow gas from the fermentor were measured continuously throughout the experiments using an Environmax NDIR Methane analyser (Liston Scientific).

Dry-matter digestion. To determine effects on the digestion of plant material in the ruminal environment, 12 nylon bags on two metal supports were inserted into the fermentor. Each bag contained a pre-weighed amount of ground Mitchell grass (approx. 1 g). At zero time and at two day intervals, two bags were removed to determine dry matter disappearance as a measure of plant digestion.

Volatile fatty acids production. Each day, at the time of replacement of fermentor liquor with fresh salts solution, a 5 mL sample of fermentor liquor was collected for determination of volatile fatty acid (VFA) production (as per Ouwerkerk and Klieve, 2001), and 1 mL to enumerate total methanogens (by Real-Time PCR) and to profile methanogen population diversity (by DGGE; Ouwerkerk *et al*., 2008).

3.2 Pen-trial experiment (*in vivo* study)

3.2.1 Animals, treatments and experimental design. The experiment was carried out at CAAS, Gatton, Qld between September and December 2011. Commercial Brahman crossbred steers approximately 10-12 months of age and weighing 240 kg live weight (at trial commencement), were sourced from "Brian Pastures" Research Station (DEEDI) via Gayndah, Qld. They were vaccinated against tick fever and also tetanus, blackleg, malignant oedema, black's disease & enterotoxaemia (5-in-1) and bovine ephemeral fever. They also received an application of Cydectin® Pour-on to reduce any parasitic burdens prior to commencement of the experiment.

The experiment was conducted as a randomised complete block design with 32 steers given four treatments (n = 8) over an 11 week duration. The treatment groups consisted of a basal hay diet of medium quality chopped Rhodes Grass (*Chloris gayana*) hay, which represented subtropical hay in northern Australian cattle production. Hay was chaffed to lengths averaging 5 cm. The control was the basal diet while the dietary treatments were this basal hay diet plus lipid containing feeds delivered at a rate of 50g lipid/kg DM intake. The supplemental lipid feeds were whole cottonseed, sunflower oil and a marine algal meal, Algamac 3050 (Aquatic Diagnostic Services International). Molasses (300g) was used as a carrier for some of the lipids to ensure palatability (Table 2). All treatments, including Controls, received the same amount of molasses.

Table 2. Diet composition of the treatments.

	Treatment diets *(g.day/animal)*			
	Control	Algamac	Sunflower oil	WCS
Rhodes Grass hay[1]	*Ad libitum*	*Ad libitum*	*Ad libitum*	*Ad libitum*
Molasses	300	300	300	300
Algamac 3050[2]	-	350	-	-
Sunflower oil	-	-	230	-
Whole cottonseed[3]	-	-	-	1300

[1] Rhodes grass hay. Offered hay (91% DM; 5.9% CP; 9.8% Ash; 41% ADF; 49.4% NDF) and residue hay (93% DM; 3.0% CP; 6.4% Ash; 50.4% ADF; 83.2% NDF)

[2] 350 g of Algamac 3050 manually mixed with a whisked with 300 ml of water and molasses in a container. Algamac (99% DM; 12% CP; 6.9% Ash)

[3] Whole cotton seed (93%DM; 19.7% CP; 4.1% Ash; 47% ADF; 63.6% NDF)

During the preliminary period, steers were exposed to various lipid supplements in a group feeding situation. This provided an environment for a quicker and greater

uptake of each supplement whereby steers learnt new feeding behaviours from each other.

The 32 steers selected were based on live-weight uniformity and temperament. These steers were allocated into individual outside covered pens and to one of the four treatment diets. Experimental week one and two were used to adapt the steers to the diets. During this adaptation period the basal diet was restricted to approximately 1.5% of their body weight which encourages the uptake of the allocated supplement. After adaptation, steers were offered the basal hay *ad libitum* at 8.00 am every morning. Quantities of hay and lipid supplement offered and refused were recorded daily. To encourage intake, the supplements were fed *ca.* 30 min prior to the hay feeding. Supplement allowances were adjusted daily for each steer based on their food intake. Hay was fed to each steer at a level estimated to provide about 15% in excess of its intake on the previous day, thereby maintaining *ad libitum* intake

All steers had continuous access to fresh water.

Steers were weighed weekly immediately prior to feeding on the same day each week.

Representative samples of the hay, supplements and refusals were taken at each feeding, and dried to constant weight at 60^{o}C to determine dry matter (DM) content. Separate samples of the feed sources and their components were taken at the same time and bulked over weeks 1-9 for chemical analysis. Samples were milled through a 1 mm screen prior to analysis for organic matter (100-ash), crude protein, neutral detergent fiber and acid detergent fiber, which were measured in triplicate (Table 2).

On day 50 of the growth study, rumen fluid was collected from all steers just prior to feeding using a stomach tube and vacuum pump under mild vacuum. The pH of the rumen fluid was measured immediately and the fluid was then strained through nylon stocking and four aliquots of 1 mL were taken, centrifuged at 13200 rpm for 10 minutes and the pellet frozen for subsequent DNA extraction and to determine methanogen numbers and diversity.

3.2.2 Methane analysis

Methane chamber design and operation. At the conclusion of the feeding trial, methane emissions were measured. Emissions were accurately measured by placing the steers in four sealed chambers, with supply and exhaust air ducts. The flow rates of air in the supply and exhaust ducts were recorded. The flow rates of the supply and exhaust ducts generated a small negative pressure inside the chambers and the air volume within the chamber was exchanged every four minutes. Four chambers were identical and chambers had dimensions of 10m long x 5m wide x 3m high (volume of 150m^3). An antechamber in each control room minimised disruption to methane concentration. Chamber surfaces were of painted concrete except for the wall dividing chambers which were aluminium framed perspex sliding doors on tracks (bolted together and sealed). The size and air flow

through the chamber meant that two steers were required to produce enough methane to analyse accurately. Therefore steers were assigned to pairs and a pair of steers were placed in each chamber, with each steer held in individual pens (2.3 x 2.3 m). These chambers enabled visual contact with the steers in the adjacent chamber.

Since only 8 animals could be measured for methane at any one time, methane measurement progressed as a complete Latin Square whereby the four treatments were measured over three rounds. Four pairs of steers were transported to the chambers and allocated to one of four chambers in the environment controlled rooms. The rooms were maintained at thermoneutral conditions i.e. relative humidity 50%-60% and ambient temperature at 24^{o}C. Within these chambers, a pair of animals within the same treatment group was allocated a chamber and housed in separate pens. Steers which did not consume supplements were not measured for emissions. Each round of methane measurement consisted of a 2 day conditioning period to minimize stress and 3 days of methane emission measurement. After each round, chambers were cleaned and recalibrated and a new group of animals allocated to the chambers. While in the chambers, animals were fed daily, on the same treatments as earlier. During feeding time, pens were cleaned and animals monitored. A fan was placed in each chamber to ensure an even mixing of the atmosphere within the chamber.

Methane measurements were taken at two hourly intervals from 7 am to 7 pm over three days, by collecting air in a 20 mL syringe (flushed out 10 times with chamber air) near the exhaust duct within each chamber. Samples at each time were taken in duplicate. Samples of air going into the room were also taken so as to calculate any background methane. The difference between the incoming and outgoing mass of CH_4 was used to calculate the amount of CH_4 generated in each chamber by the two steers. Samples were measured using a gas chromatograph at the School of Agriculture and Food Science, University of Qld, St Lucia. A sample of 0.5 mL was injected into the GC machine (GC–17A Shimadzu, Kyoto, Japan) and Methane concentration was determined by using flame ionization detector (FID) with a packed column (Parapack N, 2 mts long). The setting temperatures were 60°C (oven), 80°C (FID) and 80°C (injector). Helium was using as the carrier gas at pressure of 120 kPa. The final methane concentration was estimated by plotting a linear regression of the three points standard concentration (2, 6, and 10 ppm).

For each chamber, air flows in each exhaust and supply duct were continuously measured every 15 minutes.

Methane emission calculations. Individual animals were the experimental units for intake, live-weights and bacterial community analysis. The experimental unit for CH_4 emissions was a pair of steers (chamber) and the cumulative daily CH_4 emissions from each chamber were calculated for each day. The daily flux determined for each chamber was expressed as a proportion of DMI of the two steers combined and LW of the two steers combined. In addition methane emissions (g) were expressed as a function of average daily gain (ADG) (kg) to relate on farm production with methane emissions.

3.3 Methanogen enumeration and diversity (Real-time PCR and DGGE)

DNA extraction. Total genomic DNA (gDNA) was extracted from the 1 mL fermenter liquor (*in vivo* experiments) and pelleted ruminal fluid (pen-trial experiment) using the repeated bead-beating protocol of Yu and Forster (2005). The integrity and presence of gDNA was analysed by electrophoresis on a 1% Tris-Borate-EDTA (TBE) agarose gel, stained with GelRed and the gDNA visualised under UV light.

Denaturing gradient gel electrophoresis (DGGE). Partial 16S rRNA genes of archaea were amplified from the extracted genomic DNA by PCR. A nested PCR procedure was used with primers Arch46F (Ovreas *et al*. 1997) and Arch1017R (Barns et. al., 1994) used in the first round of amplification. The resulting PCR product was used as template in the second PCR which amplified across the variable region 3 (V3) of the 16S rRNA gene using the primers Arch344F-GC (Raskin *et. al*. 1994) and Univ522R (Amann *et al*. 1995). The PCR products were analysed on 2% TBE agarose gel using electrophoresis, stained with GelRed and the gDNA visualised under UV light. PCR products were separated on an 8% polycrylamide gel with a denaturing gradient of 30 – 60% urea/formamide by electrophoresis at 100V for 18 h in a solution of 0.5x Tris-Acetate-EDTA (TAE) buffer using DCode System (Bio-Rad, USA). The gels were stained with silver staining methods (Kocherginskaya *et al.* 2005) and DGGE images were digitalized using a scanner. The resulting banding patterns were considered the profile of the predominant methanogen species present in the samples.

Real-Time PCR. Total methanogen numbers were estimated using a real-time PCR assay (Takai and Horikoshi 2000) directed towards the domain Archaea. The primers used were Arch349F (5'-GYGCASCAGKCGMGAAW-3') and Arch806R (5'-GGACTACVSGGGTATCTAAT-3'). The probe used was 6-FAM labelled Arch516F (5'-TGYCAGCCGCCGCGGTAAHACCVGC-3'). Standards have been made by direct counting cells of *Methanobrevibacter ruminantium* ATCC35063 grown in broth culture. The cells were counted using a Petroff-Hauser Bacteria Counter. The real time PCR assay was run on a Corbett Rotor Gene 3000 under the following conditions - 1 cycle at 94^{o}C for 1 min; then 45 cycles at 94^{o}C for 10 sec, 64^{o}C for 30 sec and 30^{o}C for 1 min. The specificity of the archaeal real-time PCR was checked against the bacterial panel as described by Ouwerkerk *et al.* (2002).

4.0 Results

4.1 *In vitro* studies

4.1.1 *Spirulina* and Algamac 3050

Methane production. *Spirulina* at 3% inclusion in the diet (it was not possible to get higher concentrations into the diet due to intrinsically low lipid concentrations in this freshwater alga), had no effect on methane concentrations or other fermentation parameters. No further work was therefore undertaken with this alga.

The effect of increasing inclusion of Algamac 3050 on methane production during *in vitro* fermentation is presented in Figure 2.

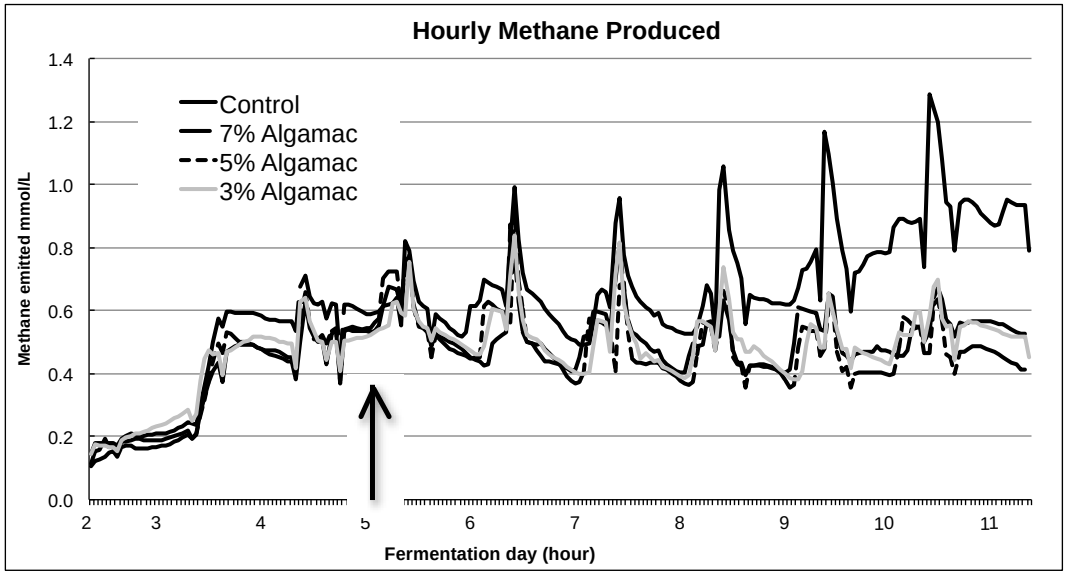


Figure 2. Hourly methane production from fermentations with 0 (control), 3, 5 and 7% Algamac3050. Supplement was added to the feed from day 5 onwards (arrowed).

Algamac reduced methane generation in a sustained manner over the entire period of fermentation. This effect doesn't appear to be dose dependant with 3% oil reducing emissions by a similar amount to 7% oil. This tends to suggest that other properties of the algae, not just oil content, may be involved.

Effect on dry-matter digestion. The effect of Algamac3050 inclusion on forage Dry Matter utilization is presented in Figure 3.

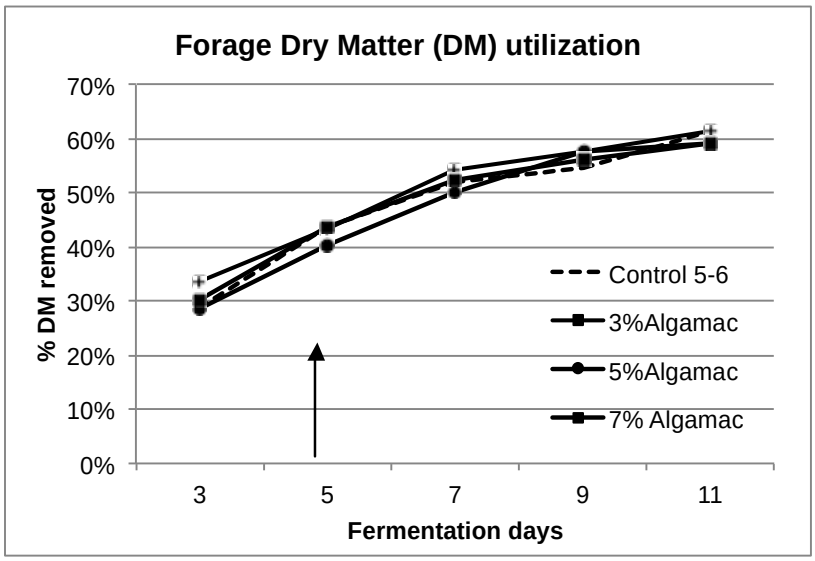


Figure 3. Percent Dry Matter reduction of Mitchell grass in fermentation with 0 (control), 3, 5 and 7% Algamac 3050. Arrow indicates first day of algae inclusion.

No differences in dry matter digestion were observed between the supplemented fermentations and the controls. Thus, there is no negative effect on the fibrolytic activity evaluated by dry matter disappearance at the three different levels of Algamac inclusion.

VFA production. Total VFA produced each day for control runs and Algamac treatments at 3, 5 and 7% are presented in Figure 4. There did not appear to be any significant effect of algae supplementation on total VFA production, however the acetic:propionic acid ratios were changed as seen in Figure 5. Thus, the supplementation of Algamac changed the rumen fermentation, resulted in greater propionic to acetic acid proportions at all dose rates. As the production of propionic acid is an alternative hydrogen sink to methanogenesis, this may be a significant observation.

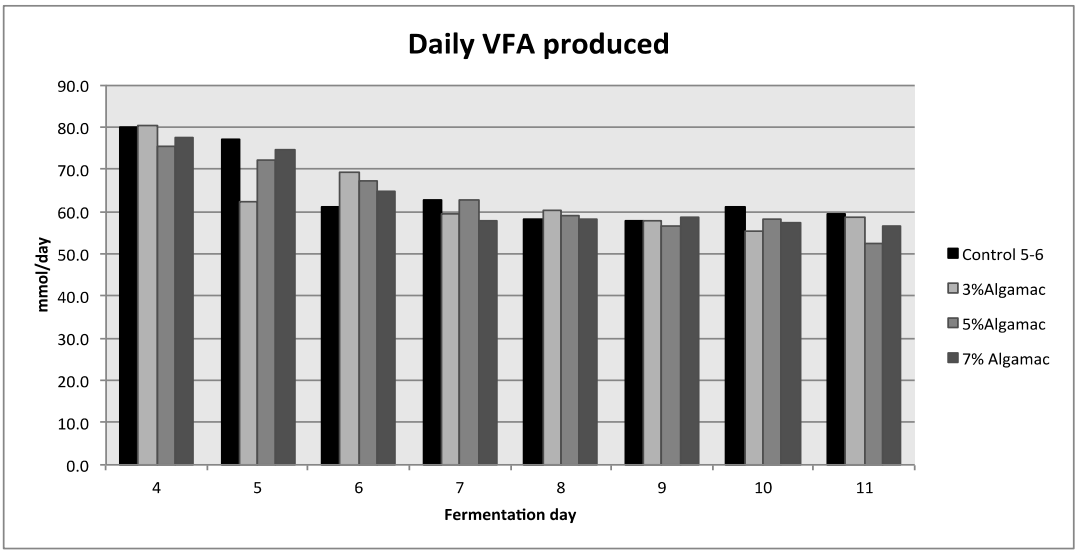


Figure 4. The effect of Algamac3050 on total daily VFA production (mmol/d).

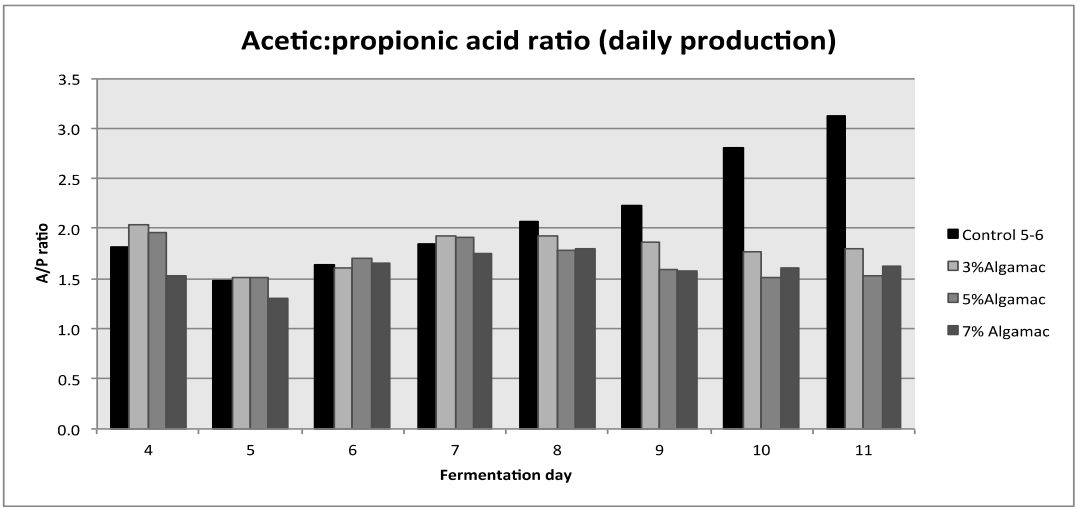


Figure 5. The effect of Algamac 3050 supplementation on the daily acetic:propionic acid ratios.

Methanogen population density and diversity. The total numbers of methanogens (Archaea), as determined by real-time PCR, present in the fermentor are presented in Figure 6.

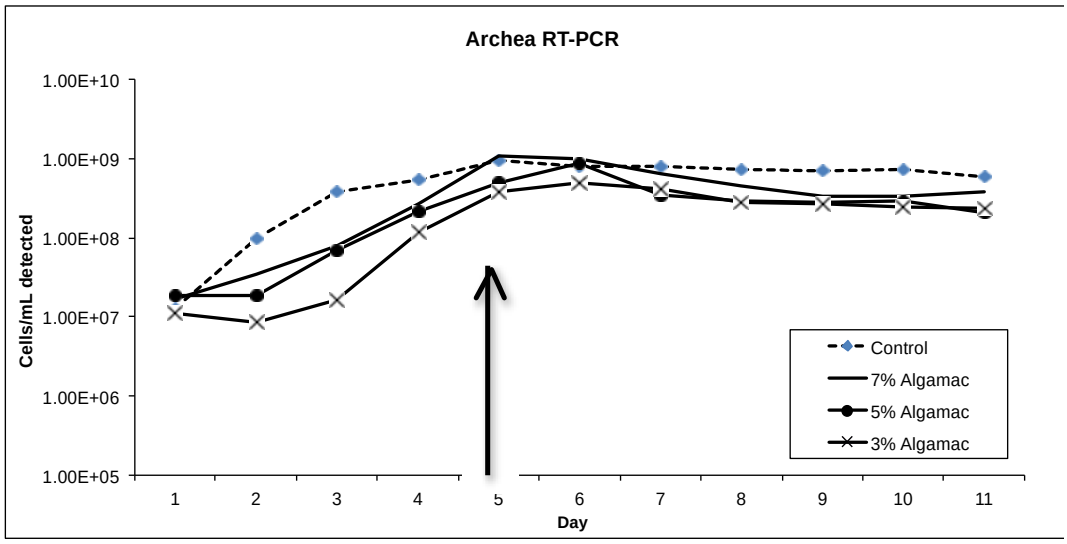


Figure 6. Cell equivalents of total archaeal populations in fermentation liquor. Lipid supplement Algamac 3050 was added to the diet from day 5 onwards (first day of addition indicated with an arrow).

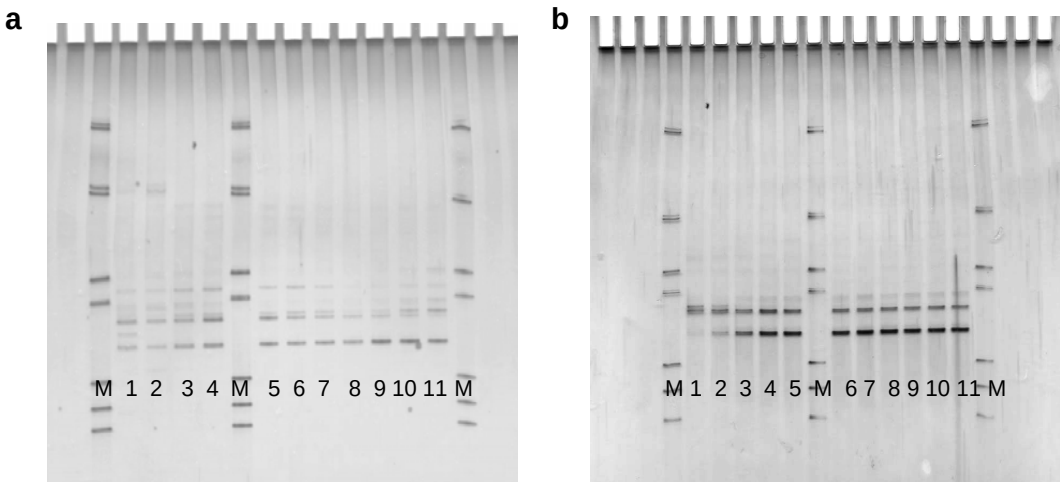


Figure 7. DGGE profiles of the archaeal community in fermentors without lipid inclusion (a) and with 7% Algamac 3050 addition (b). M – Markers. Numbers correspond to day of fermentation.

Methanogen numbers in control fermentations increased gradually during the first four days of fermentation to stabilise at just below 10^9 cell equivalents per mL of fermentor liquid (Figure 6). This community density was maintained for the remainder of the fermentation. The inclusion of 3, 5 and 7% Algamac 3050 in the diet appeared to have minimal effect on methanogen numbers or methanogen community structure (profiling using DGGE) which was not altered throughout any of the fermentations. Two examples are shown in Figure 7.

4.1.2 Canola oil

Methane production. The effect of 5 and 7% inclusion of canola oil on methane emissions during *in vitro* fermentation is presented in Figure 8.

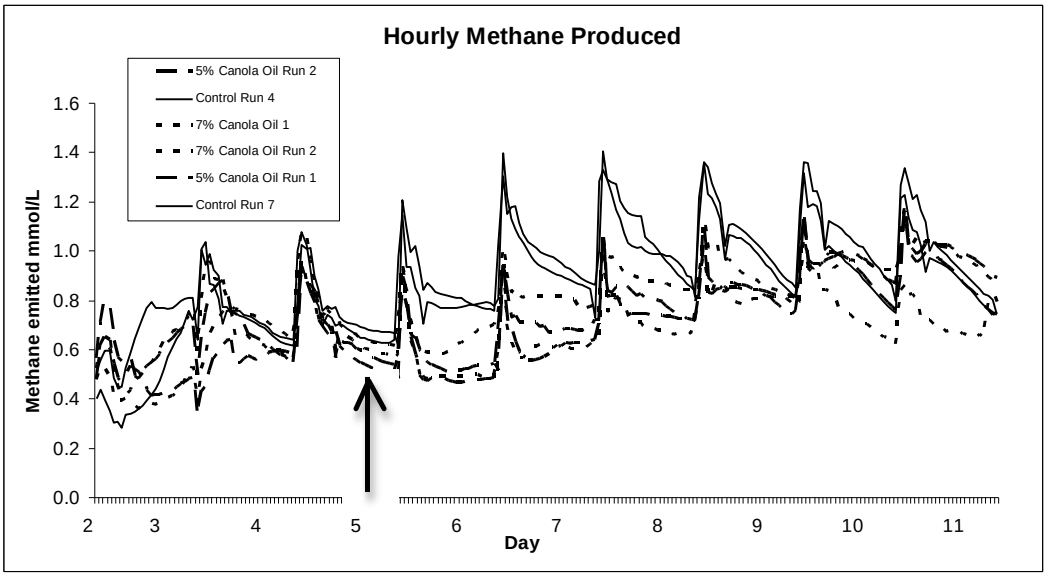


Figure 8. Hourly methane production from fermentations with 0 (control), 5 and 7% canola oil. Supplement was added to the feed from day 5 onwards (arrowed).

Introduction of 5 and 7% canola oil reduces the peak and minimum emissions in the daily cycle, by a similar amount, however this reduction is not great and was not sustained. Methane emissions slowly rose over 4 to 5 days to be similar to the control fermentations by the end of the fermentation period. It appears that the microbial populations rapidly adjust to canola. Unlike other oils tested, higher levels of inclusion of canola oil had an apparently transient impact in lowering methane generation and would not appear to be a good candidate as a feed supplement for lowering methane emissions.

4.1.3 Safflower oil

Methane production. The effect of increasing concentrations of safflower oil on methane production during *in vitro* fermentation is presented in Figure 9.

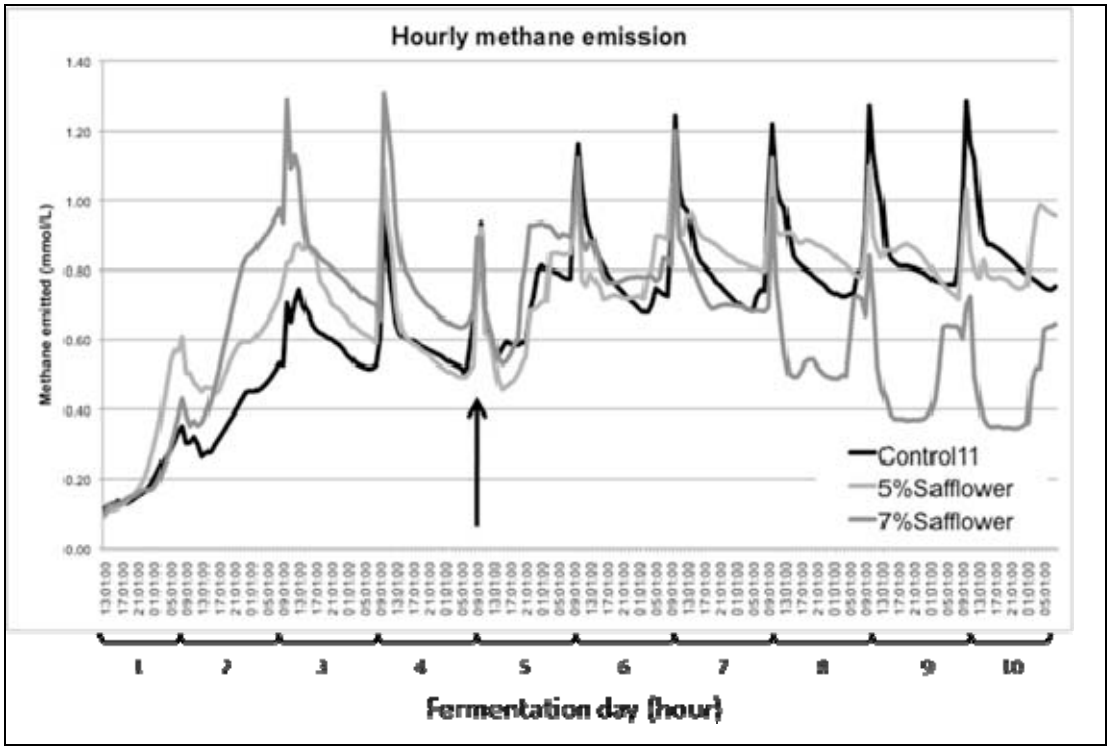


Figure 9. Hourly methane production from fermentations with 0 (control), 5 and 7% safflower oil. Supplement was added to the feed from day 5 onwards (arrowed).

In *in vitro* fermentation, the higher level (7%) of inclusion of safflower oil reduced the total methane emitted. For the intermediate safflower supplementation, no reduction was detected and similar emission curves were observed between control runs and 5% inclusion. With these results, a lower inclusion rate (3%) was not considered worthy of investigation.

Effect on dry-matter digestion. The effect of safflower oil inclusion on forage Dry Matter utilization (*in vitro* fermentation) is presented in Figure 10.

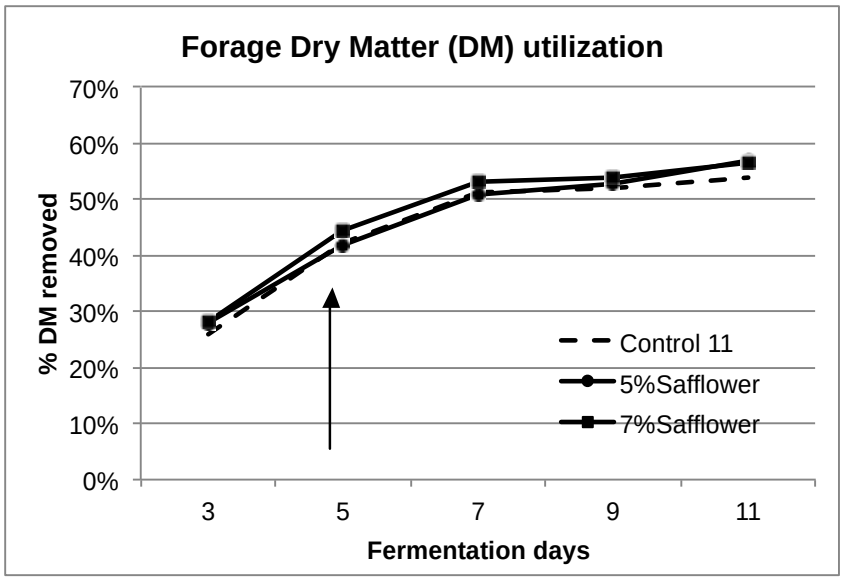


Figure 10. Percent Dry Matter reduction of Mitchell grass in fermentation with 0 (control), 5 and 7% safflower oil. Arrow indicates first day of oil inclusion.

There was no effect on fibrolytic activity or dry matter digestion for safflower oil inclusion. At day 11 of the fermenter run, nearly 55% of the plant material was digested in the control and safflower runs. These results suggested that the intermediate and highest safflower oil might be incorporated in the diet of ruminants without affecting the digestion of plant fibre.

VFA production. Total VFA produced each day of fermentation for control and safflower oil at 5 and 7% are presented in Figure 11. At the end of fermentation (day 11), the total VFA produced (mmol/d) were slightly lower comparative to the control for both concentrations of oil. However, the proportion of propionic acid was affected markedly by 7% oil inclusion (Figure 12). The acetic:propionic acid ratio was lower in 7% (A:P ratio 2.1) than the 5% oil inclusion (A:P ratio 3.3).

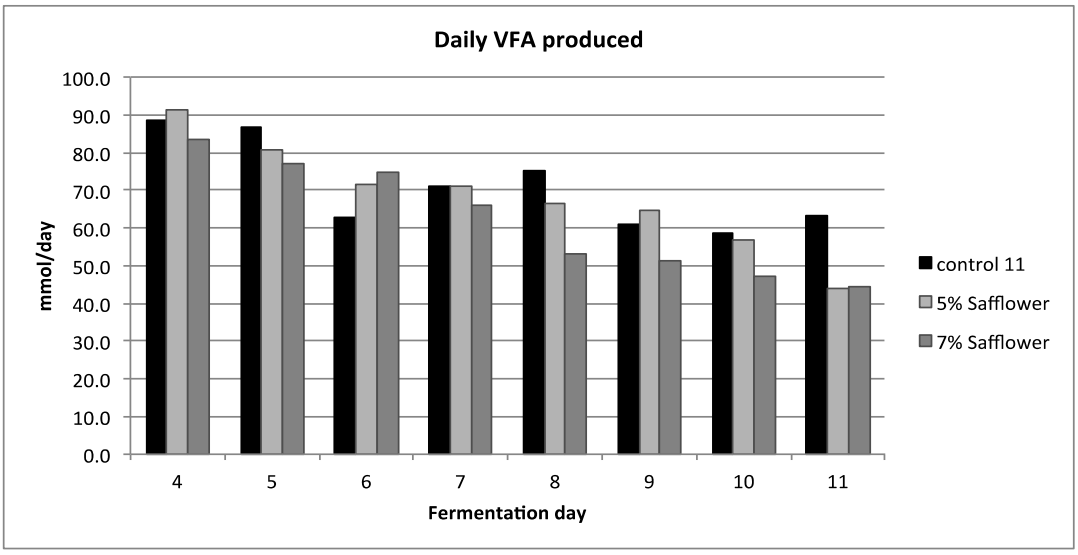


Figure 11. The effect of safflower oil on total daily VFA production (mmol/d).

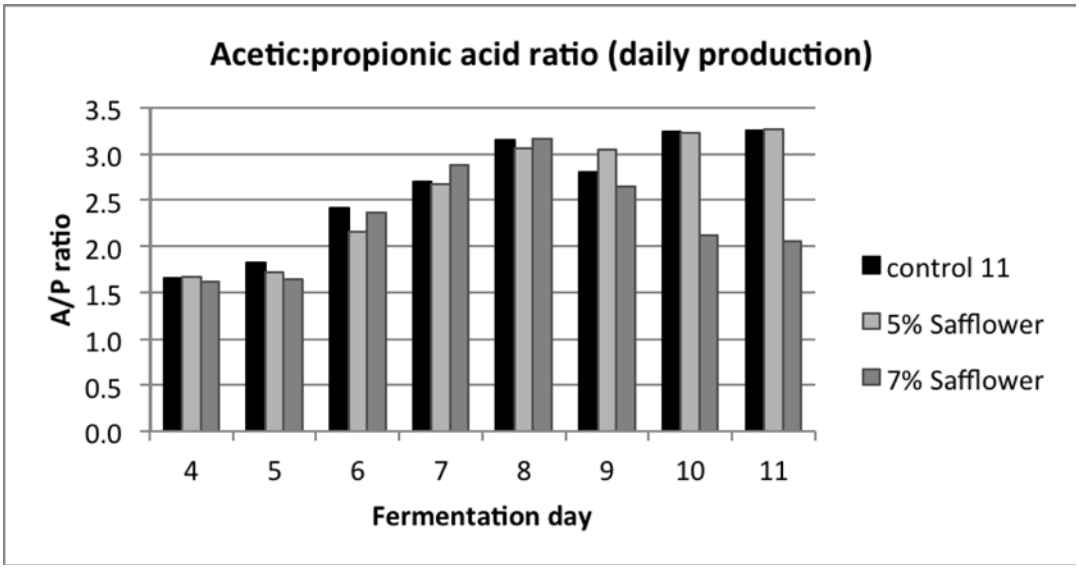


Figure 12. The effect of safflower oil supplementation on the daily acetic:propionic acid ratios.

Methanogen population density and diversity. The total numbers of methanogens, as determined by real-time PCR, present in the fermentor are presented in Figure 13.

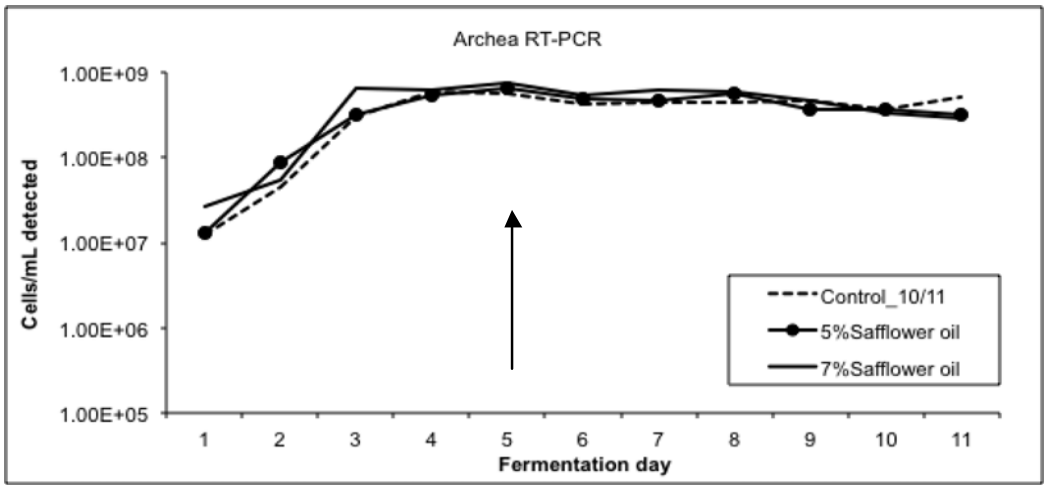


Figure 13. Cell equivalents of total archaeal populations in fermentation liquor in fermentation with 0 (control), 5 and 7% safflower oil. Lipid supplement was added to the diet from day 5 onwards (first day of addition indicated with an arrow).

The numbers of methanogens in the *in vitro* fermentation was not affected by the inclusion of safflower oil. The methanogen population maintained and remained stable at below 10^9 cell equivalents per mL from day 4 in the control and safflower oil treatments. The inclusion of safflower oil in the diet appeared to have no effect on methanogen numbers.

The community structure, visualized using DGGE, showed that the diversity of the archaeal community did not change throughout the fermentation in the control or safflower oil treatments (Figure 14).

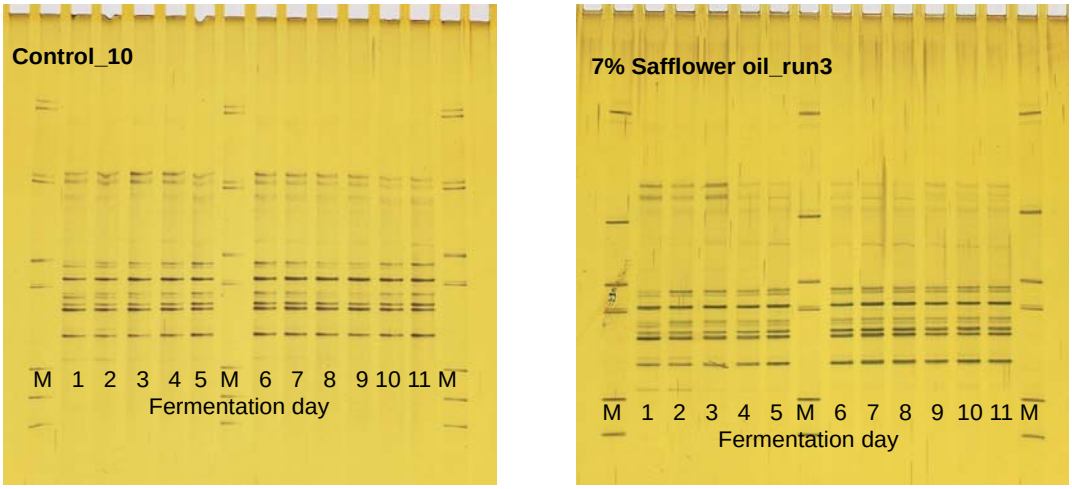


Figure 14. DGGE profiles of the archaeal community in fermentors without lipid inclusion (a) and with 7% safflower oil addition (b). M – Markers. Numbers correspond to day of fermentation.

4.2 Pen study: Effect of lipid supplements on the growth and methane production of steers

4.2.1 Cattle growth and methane production. The initial two week supplement introduction period, showed a reduction in DM intakes due to the restriction of hay intake to encourage the various supplement uptakes. Molasses was readily taken up by steers. The prescribed lipid concentration in diet was 50 g/kgDMI as a higher concentration of lipids is associated with a reduction in hay intake. During this introductory period, the uptake of sunflower oil and whole cottonseed reached the prescribed intake. The sunflower oil treatment group maintained the desired intake of oil however by week 3 hay intake reduced and there were high quantities of refusals (around 1 kg) in most of the steers in this group. When hay intake was reduced, oil was provided at a lower quantity so that the prescribed lipid concentration was maintained. After an initial high intake of whole cottonseed, intake of whole cottonseed for five steers was variable with two steers consuming very little. Supplement intake for Algamac was well below the desired level, even when the supplement mixture of molasses and water was adjusted to change the sweetness to mask the strong algal taste. The favoured consistency of the algal mixture was a runny paste.

Weekly live-weights in steers fed the various dietary lipid treatments are presented in Figure 15 and Table 3. All treatments showed an initial decline in live-weight which corresponded to the restriction in hay intake over the first two weeks. On the hay only basal diet, the hay control maintained live-weight over the 11 week growth study. Steers receiving whole cottonseed showed the greatest response in live-weight gains, even though supplement intake was variable. The higher live-weight gains with whole cottonseed, relates to the higher nutritional quality, in particular a higher N content. Algamac supplementation also resulted in steady live-weight response even though supplement uptake was relatively poor. The higher than expected live-weight response reflects the better nutritional content in Algamac. Sunflower oil uptake was always to the desired level due to the addition of molasses, however the lipid level had to be continuously adjusted due to the negative effect of the oil on hay intake.

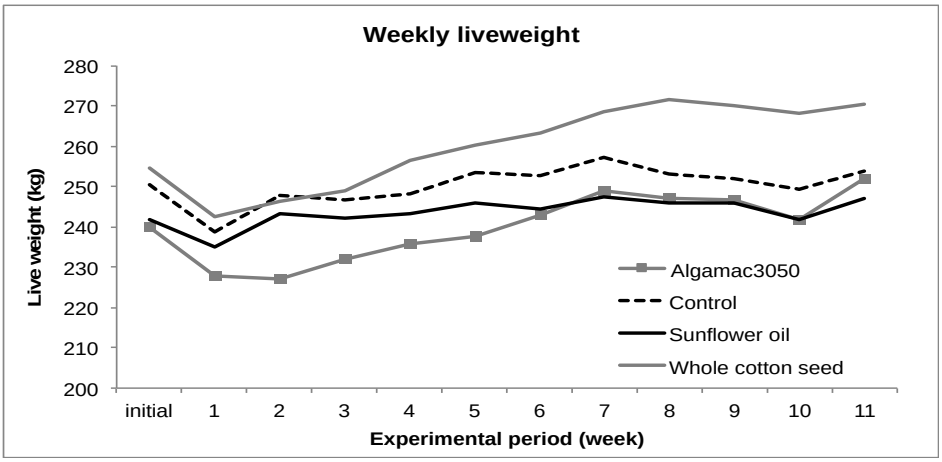


Figure 15. Live-weight gains of the dietary treatments over the 11 week experimental period.

Plotted data relating to methane production as a function of the DMI and LW are shown in Figures 16 and 17. No error bars were added to these graphs to prevent cluttering of the data. It was noted in these graphs that there was considerable variability within and between animals. Taking into account the differences in live-weight between steers, methane emissions were greatest in the control and steers supplemented with whole cottonseed. Taking differences in dry matter intake into account, methane emissions were higher in the control steers. The values calculated in this study are similar but slightly higher than that found in other studies (Beauchemin and McGinn 2006: Beauchemin *et al*. 2007; Beauchemin *et al.* 2009). This is likely to be due to the tropical pasture type and the breed of cattle used in this trial. When methane emissions were related to dry matter intake, the supplementation of lipids did reduce methane emissions, and was most evident in the sunflower and Algamac treatments. There was diurnal variation in methane production. The major peaks occurred 10 hours after feeding in all treatments but not as extreme in the sunflower and Algamac treatments. Lowest levels of methane production occurred just before feeding. Differences in methane production as a function of the steers live-weight, related to a 21% reduction in the Algamac group and 19 % reduction in the sunflower group (Table 3).

Data relating methane production per animal (g) to average daily gain (ADG) (kg) is found in Table 3. Differences between treatments were greater when taking into account the steers ADG with the lowest methane emissions occurring in the Algamac and whole cottonseed treatments. This trend is similar to other studies whereby methane production per unit ADG is reduced as growth rates increase (Kurihara *et al.* 1997). These treatments were four times lower than the control. In addition is the fact that due to the greater live-weight gain, the number of days to market would be reduced therefore further reducing total overall emissions. What is of particular interest is that the Algamac treatment steers did not consume even close to the prescribed level, however, they still showed promising results. It therefore appears that the response in methane production in this treatment group is not dose related, in agreement with the *in vitro* results.

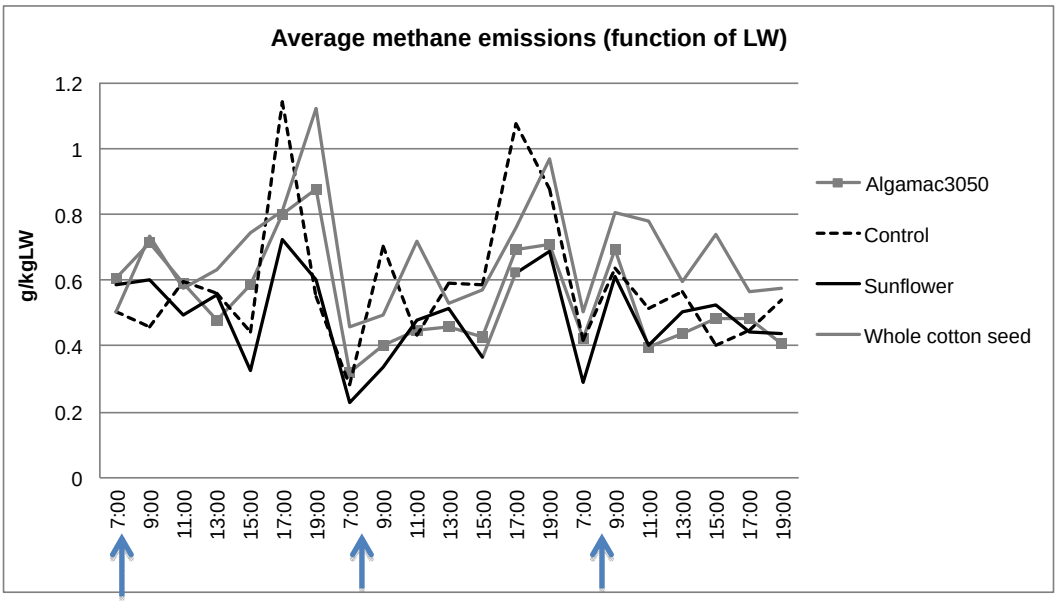


Figure 16. Effect of lipid supplements on methane production as a function of live-weight in steers given a basal diet of medium quality Rhodes Grass hay *ad libitum*. Lines represent averages (three periods by two animals; n = 6) for each treatment. Arrows indicate when daily feeding occurred.

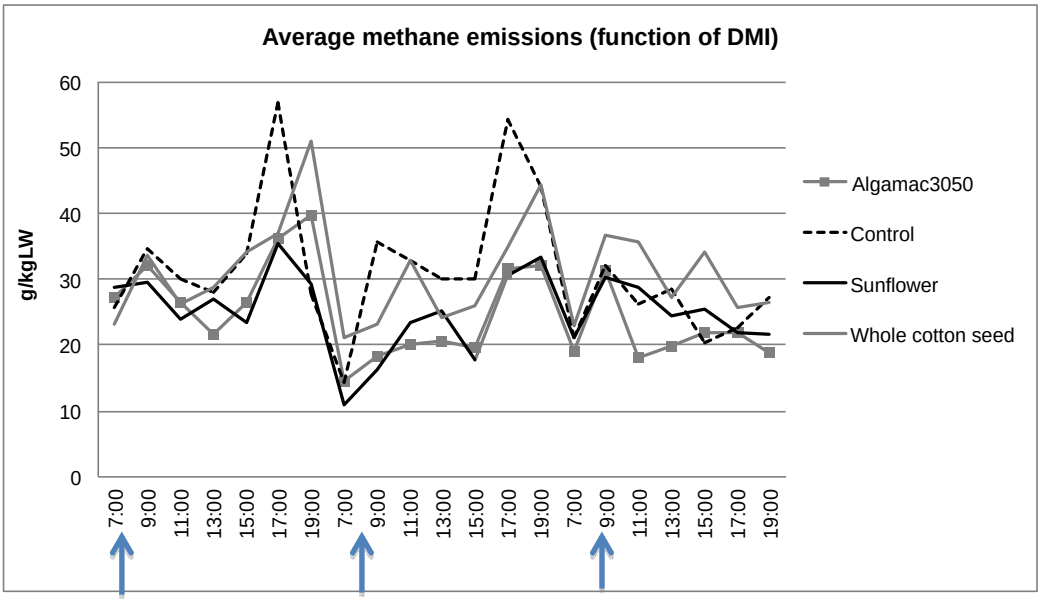


Figure 17. Effect of lipid supplements on methane production as a function of dry matter intake in steers given a basal diet of medium quality Rhodes Grass hay *ad libitum*. Lines represent averages (three periods by two animals; n = 6) for each treatment. Arrows indicate when daily feeding occurred.

Table 3. Effect of lipid source on body weight, intake and methane emissions of steers.

	Treatment			
	Control	Sunflower oil	Whole Cotton Seed	Algamac
End experimental LW (kg)	253.7	246.8	270.5	252.1
LW change (kg)	3.1	5.2	15.7	12.1
DMI (g)	5077	5093	5819	5468
Methane g/kg LW	0.587	0.493	0.676	0.545
Methane g/kg DMI	31.3	25.2	30.9	24.7
Methane g/kg ADG	3851.69	1871.55	919.55	913.84
Ruminal pH	6.9	6.9	7.1	7.0

4.2.2 Methanogen population density and diversity. There was a larger population density of methanogens (Archaea) in the whole cottonseed group compared to other treatments (Figure 18), which may have been due to a better nutritional profile of this diet, providing improved growth conditions for these microbes. There was little differences in population numbers of the other treatments but considerable between animal variability did occur.

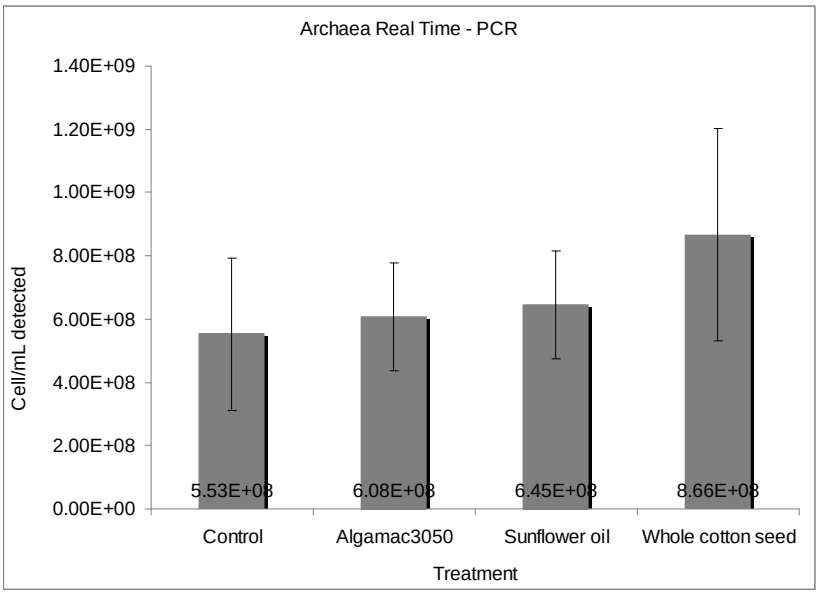
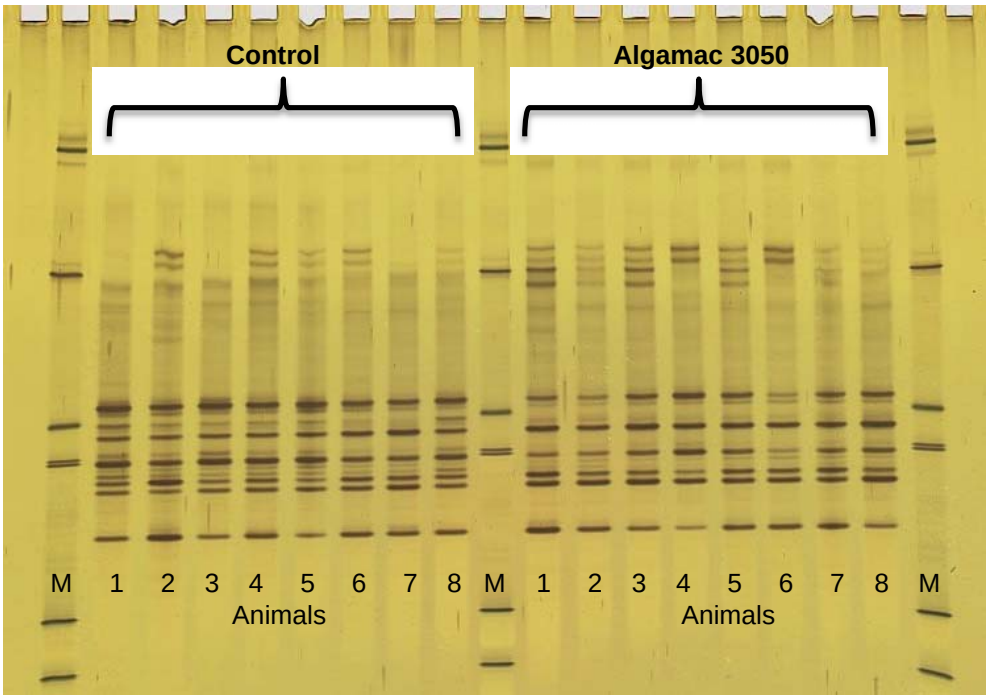


Figure 18. Effect of lipid supplements on methanogen populations in steers given a basal diet of medium quality Rhodes Grass hay *ad libitum*. Bars show standard deviations for each treatment.

When individual steer community profiles (by DGGE) were compared, there were very few differences with respect to the various treatments, or to individual steer variability (Figure 19). These are similar results to the *in vitro* methanogen profiles. The only changes seen in the profile is in the Algamac treatment group with individual differences relating to the low acceptance of this supplement (Table 4). When Algamac acceptance was below 100g/day, darker bands were present in the upper part of the profile. These bands were less prominent with a higher intake of Algamac. It therefore appears that changes in methane production were not related to changes in the community structure of methanogens or with the populations of methanogens.



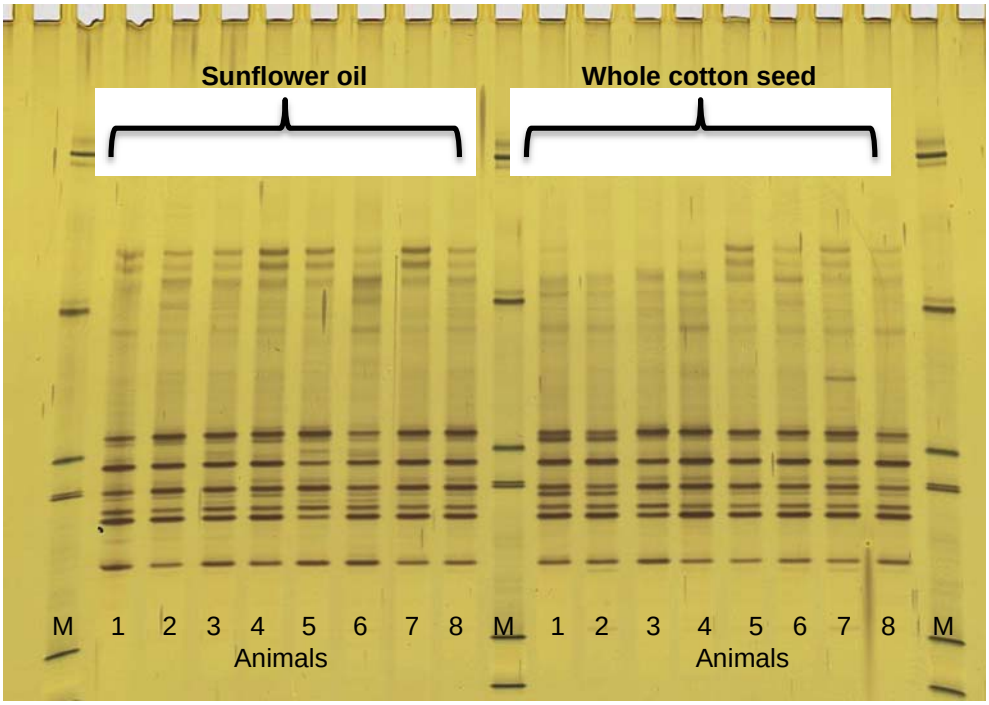


Figure 19. DGGE profiles of the archaeal community in rumen fluid of control group steers and those supplemented with Algamac, sunflower oil and whole cotton seed. M – Markers. Numbers correspond to individual steers within each group.

Table 4. Corresponding DGGE lane (figure 19), animal ID and total Dry Matter Intake and supplement intake on week 6 of the experimental period.

Gel 1-figure 19. Control and Algamac 3050

Treatment	DGGE lane #ID	Animal #ID	Dry Matter Intake (g.day/animal)	Supplement intake (g.day/animal)
Control	1	1668	4380	0
	2	1652	3570	0
	3	1646	4740	0
	4	1673	4380	0
	5	1642	4740	0
	6	1666	4650	0
	7	1671	4560	0
	8	1660	4740	0
Algamac 3050	1	1670	4790	50
	2	1644	4854	114
	3	1665	4840	90
	4	1638	4430	50
	5	1672	4437	57
	6	1653	4710	60
	7	1643	4800	150
	8	1658	4860	120

Gel 2-figure 19. Sunflower oil and Whole Cotton Seed

Treatment	DGGE lane #ID	Animal #ID	Dry Matter Intake (g.day/animal)	Supplement intake (g.day/animal)
Sunflower	1	1654	3730	160
	2	1640	4000	160
	3	1637	4360	160
	4	1651	4180	160
	5	1669	3820	160
	6	1659	4810	160
	7	1674	4810	160
	8	1648	4900	160
Whole Cotton Seed	1	1667	4833	93
	2	1639	5688	948
	3	1661	4687	37
	4	1664	5628	888
	5	1657	5865	1215
	6	1656	4814	74
	7	1650	5769	1028
	8	1675	5207	467

5.0 Discussion / Conclusion

Ruminal archaeal communities of rumen methanogens are responsible for hydrogen metabolism and methane (CH_4) production. Environmentally, CH_4 is an important greenhouse gas and its emissions from ruminants (beef, dairy and sheep) has been estimated to be between 14 and 50% of the total greenhouse gas emissions in countries like Australia and New Zealand (Leslie *et al.*, 2007). For this reason, ruminal methanogens are being widely studied in order to mitigate emissions by finding antimethanogenic agents or other nutritional strategies. Strategies such as nutritional management by adding additives to the diet (lipids, ionophores, yeast, plant extracts or organic acids) and biocontrol by bacteriophage or bacteriocins have been recently summarized by Beauchemin *et al.* (2008) and McAllister & Newbold (2007). From a nutritional view point, not only are methanogens important for the loss of feed energy but also they maintain a low pressure of hydrogen in the rumen, which would otherwise produce inhibitory effects on microbial metabolism (Janssen and Kirs 2008). In this communication, we report on the effect on methane emission and rumen methanogens of the inclusion of lipids in ruminant diets were evaluated on *in vitro* and *in vivo* experiments.

In previous work (see Final Report for NBP.352, MLA), using similar *in vitro* methodology to this report, other sources of lipids were evaluated. Whole desiccated coconut flesh, purified coconut oil and ground whole cottonseed reduced methane production and effected rumen function in a dose dependent manner. There was a reduction in methane emissions with ground cottonseed at 7% oil inclusion in the diet, which was similar to 7% coconut oil. However, while measurable reductions in methane occurred, the impact of cottonseed on dry matter digestion and VFA production was less adverse than with the coconut products. Cottonseed inclusion at 5% oil content of the diet appeared to have a minimal impact on fermentations including on the reduction in methane emissions. Coconut oil at 3% inclusion had very little impact on methane production but did alter VFA production and reduced fibrolytic activity slightly. At 7%, coconut oil reduced methane generation by 80% but depressed VFA production and fibrolytic activity. Whole desiccated coconut flesh was similar to purified coconut oil. While methane was reduced the impact of cottonseed on dry matter digestion and VFA production was less adverse than with the coconut products.

The impact of Algamac, canola oil and sunflower oil, at increasing levels of inclusion, on methane generation and rumen function was tested in the current project. Algamac 3050 at all levels of inclusion (3, 5 and 7%) reduced methane emissions whilst appearing to not affect rumen microbial population dynamics, including the methanogen population. There were no differences in dry-matter digestion or the numbers of methanogens or methanogen population structure. All levels of Algamac inclusion had similar effects, suggesting that the mechanism that lowers methane with algal oils is different to that of other oils, which directly impact on the microbial ecosystem at higher levels of inclusion. Canola oil at 7% inclusion appears to only transiently reduce methane generation and would not appear to be a good candidate as a feed supplement for lowering methane emissions. *In vitro* studies on the safflower treatments show it acts similar to the cottonseed and coconut oils in that it reduces methane production in a dose dependant and sustained manner. Where, 7% safflower oil inclusion significantly reduced methane production, 5% was not effective.

No differences in dry matter digestion were observed between the supplemented lipids and the controls. Over the eleven days of the *in vitro* fermentation, there was

no negative impact on dry matter disappearance at any levels of inclusion of Algamac or safflower oil. The total VFAs produced in safflower oil inclusions were slightly lower compared with the controls, and both levels of oil inclusion (5 and 7%) had a similar concentration of VFAs. However, the proportion of propionic acid increased only at 7% of oil inclusion. Thus, Algamac supplementation can be an interesting alternative in ruminant diets in order to increase the intake of organic matter, increasing the proportion of propionic acid while decreasing methane emission and not affecting fibrolytic activity up to the maximal inclusion rate tested, at 7%.

The inclusion of whole cottonseed or Algamac in the diet, caused live-weight gains in steers of 15 kg and 12 kg respectively over an 11 week period. Sunflower oil, although readily consumed by the steers had a negative impact on hay consumption, most likely by reducing ruminal fibre digestion. Concomitant with the increased productivity of whole cottonseed supplementation, there was no overall change in methane emissions per head, either on a live-weight basis or by taking dry matter intake into account. However, there was a 22% reduction in methane emission/head when Algamac was used as a supplement. In addition, there was a substantial decrease in methane emitted per unit of production (3851 g CH_4/Kg ADG to 913 g CH_4/kg ADG) when either whole cottonseed or Algamac was used as a supplement. Palateability issues arising from the use of Algamac was evident throughout this trial and supplement intakes were well below the desired level. There were also problems regarding the consistent uptake of whole cottonseed for over half the steers in this treatment group. Regardless, the use of whole cottonseed and Algamac (even at suboptimal concentrations) increased the growth rates of cattle and decreased the methane produced per unit of product (live-weight gain).

The mechanisms by which methane production is reduced by the supplementation of these lipids is unclear. Methanogen populations increased with whole cottonseed supplementation which was likely to be related to the greater nutritional plane within the rumen. Community composition of methanogens was very similar for all steers regardless of treatment and there was little individual variability. Small changes were found in profiles within the Algamac treatment and these differences related to the very low uptake of this supplement. Limitations with the use of Algamac at the current time would be cost and also palatability, however, the impact on emissions and liveweight gain with quite low intake, suggest that it should be investigated further in the future.

It is therefore recommended that the use of whole cottonseed and Algamac at a rate of 5% lipid in the diet of cattle fed these types of pasture. This will increase live-weight gain in cattle and reduce methane emissions per unit of product. Methane emission reduction will also be evident through the reduction in days-to-market due to the higher live-weight gain. Unlike many other feeding technologies (where reduction per unit product are often accompanied by increases per head) the advantages of increased production and lowered emissions per unit of production will reduce overall on farm methane emissions and will not require a reduction in herd numbers to offset any per head or per unit area increases in emissions (as is often the case with other strategies).

Individual economic evaluation of on-farm profitability should be undertaken with the relationships between methane reduction per unit of growth, cost of supplement, and uptake of supplement incorporated in the modelling of on-farm emissions and in assessing where reductions or carbon savings could be made. The next step in this research should be to feed the supplements at a practical on-farm level and take comparative herd-based methane measurements to calibrate what reductions could be achieved in a true farming situation.

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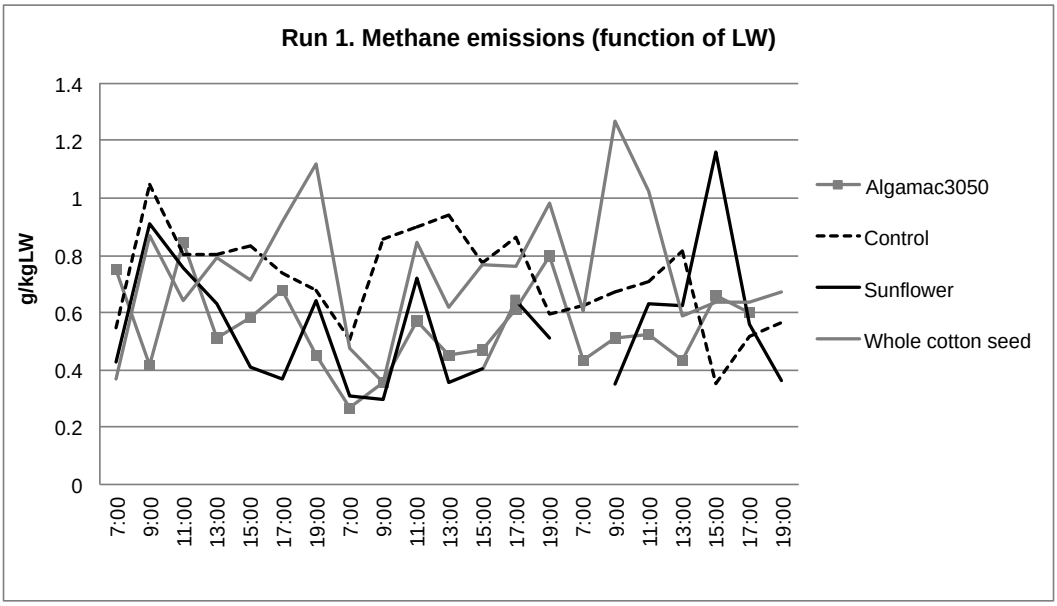
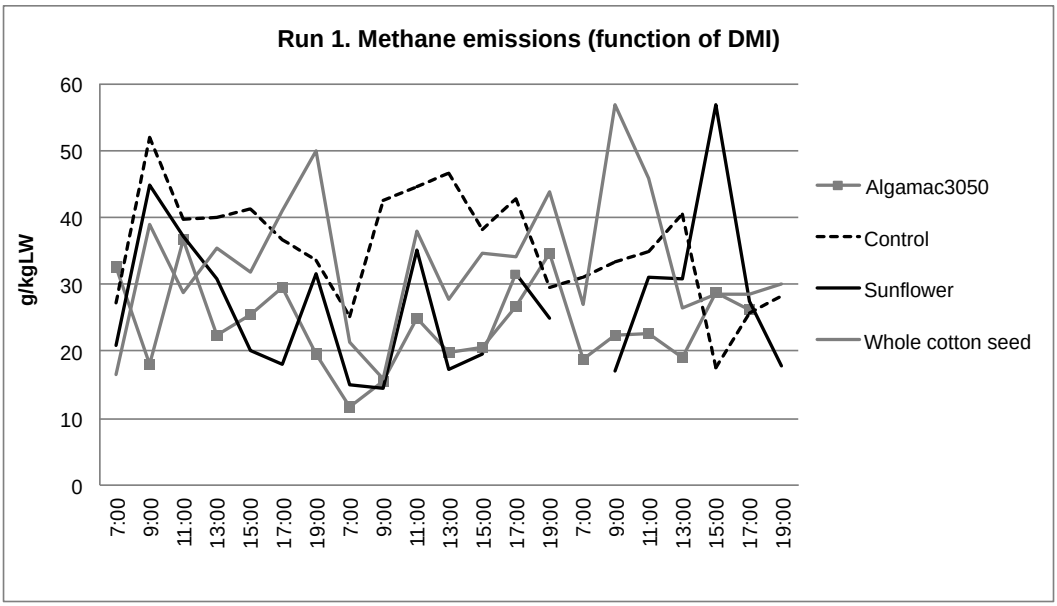
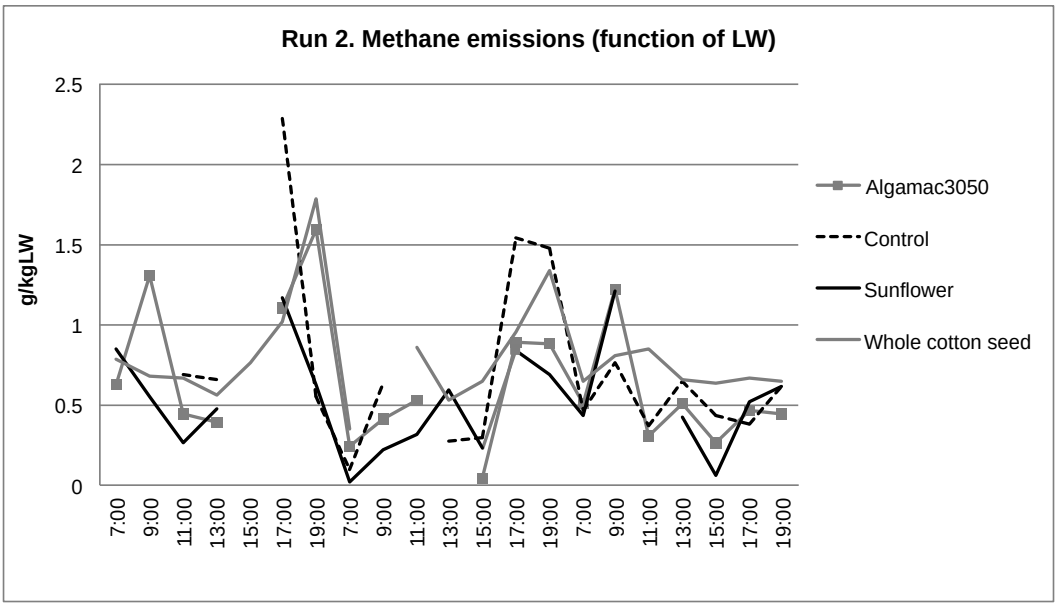
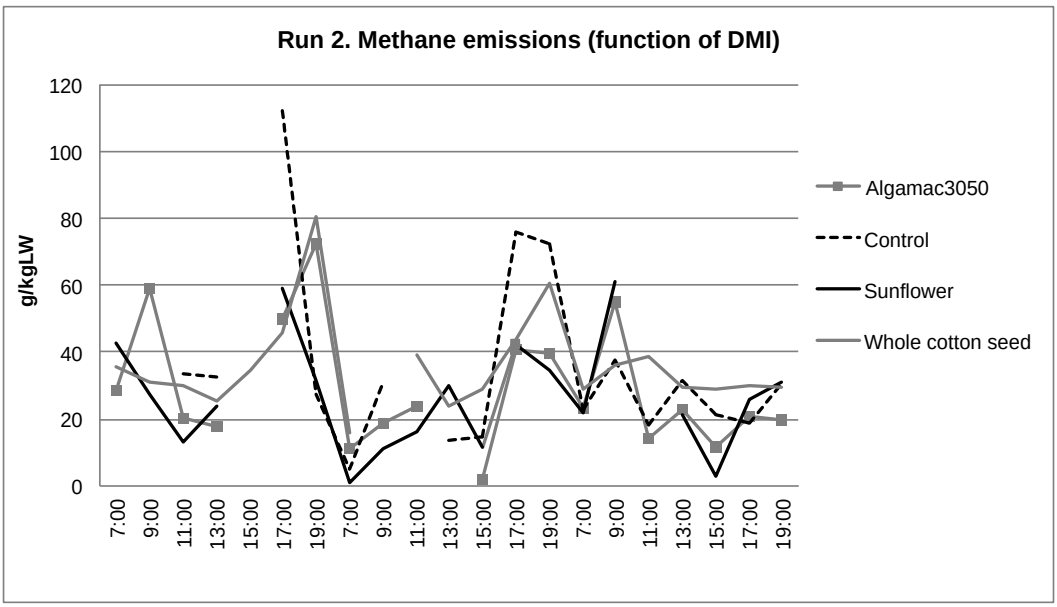
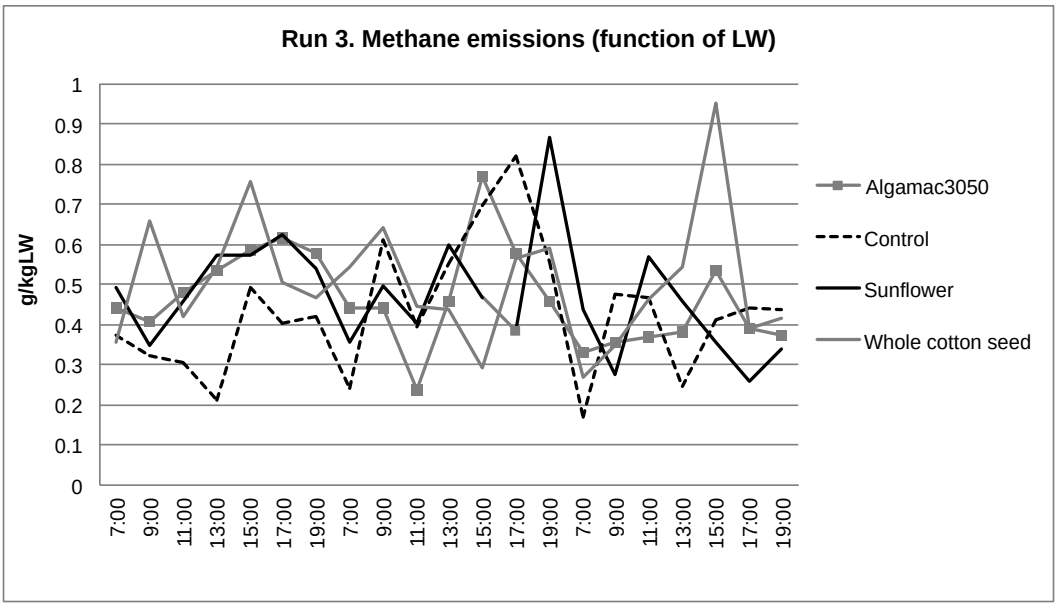
7.0 Appendix 1. Results of *in vivo* emissions for each period with animals in chambers.

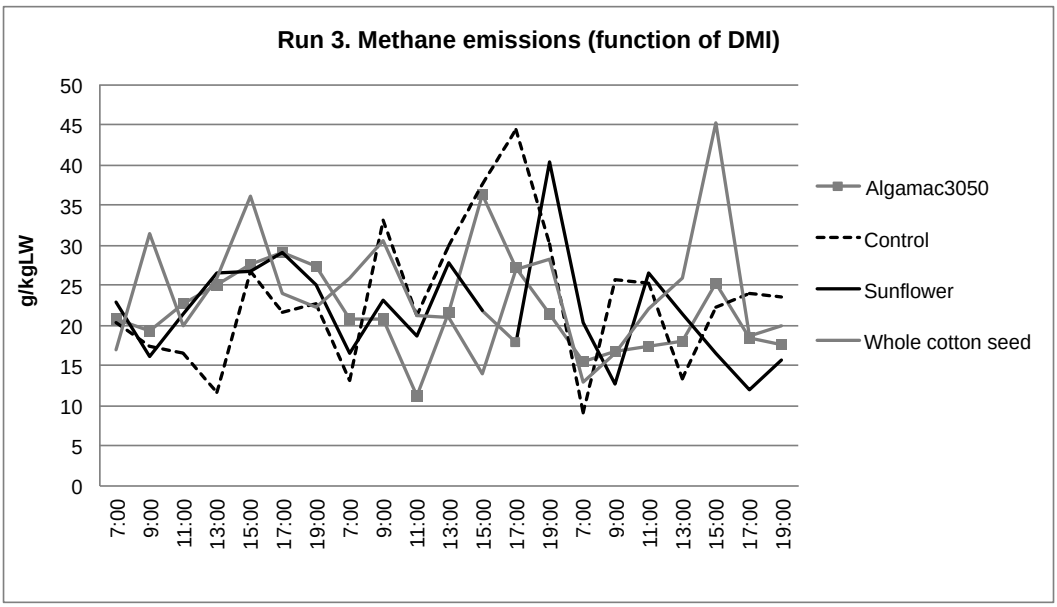





Run 2. Methane emissions (function of LW)
g/kgLW
2.5
2
1.5
1
0.5
0
7:00
9:00
11:00
13:00
15:00
17:00
19:00
Algamac3050
Control
Sunflower
Whole cotton seed


Run 2. Methane emissions (function of DMI)
g/kgLW
120
100
80
60
40
20
0
7:00
9:00
11:00
13:00
15:00
17:00
19:00
Algamac3050
Control
Sunflower
Whole cotton seed


Run 3. Methane emissions (function of LW)
g/kgLW
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
7:00
9:00
11:00
13:00
15:00
17:00
19:00
Algamac3050
Control
Sunflower
Whole cotton seed


Run 3. Methane emissions (function of DMI)
g/kgLW
50
45
40
35
30
25
20
15
10
5
0
7:00
9:00
11:00
13:00
15:00
17:00
19:00
Algamac3050
Control
Sunflower
Whole cotton seed