Australian Government
Department of Agriculture

CSIRO

The University of Western Australia

mla
MEAT & LIVESTOCK AUSTRALIA

final report

Project code: B.CCH.6530

Prepared by: Professor Philip Vercoe
University of Western Australia

Date published: August 2015

ISBN: 9781741919462

PUBLISHED BY
Meat & Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

The mechanism of antimethanogenic bioactivity of plants in the rumen

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

Acknowledgements

Dr Gavin Flematti, Research Fellow for knowledge and expertise on chemical analyses, fractionation; Mrs Azizah Algiebry PhD student and Mrs Sammar Alsaedi MSci student, School of Chemistry and biochemistry for obtaining fractions and compounds for testing. Mr Bidhyut Banik for work on *B. pelecinus*

Executive summary

We have examined several aspects of plant bioactivity against methane production by rumen microbes. We have isolated and examined the effect of plant fractions and purified compounds in mixed rumen populations (batch culture or RUSITEC), as well as in pure culture of rumen methanogens. In these systems, we measured the effect on rumen activity, microbial end-products, shifts in microbial populations, persistency and possible adaptation to the bioactive additive, as well as the mechanism of the effect.

While we have isolated several potent antimethanogenic fractions amongst additional plants that were likely to be tannins, we have found that extractable non-tannin compounds are responsible for the antimethanogenic effect in the bioactive plants *Eremophila glabra* and *Biserrula pelecinus*. Using a bioassay-guided fractionation, we have isolated multiple potent antimethanogenic fractions from these. Fractions from *B. pelecinus*also had a potent effect against key methanogens, with one fraction producing aneffect that was close to cidal.

Finally, we have found antimethanogenic properties amongst several commercial plant bioactive products,including grape marc, L, C and G. In the later, we confirmed that the antimethanogenic effect may persist over two weeks without disrupting normal rumen fermentation. These compounds had a very potent effect in pure culture of methanogens and further workon these isneeded.

Table of Contents

8. Background

In the past, we have identified a range of plants (Australian native shrubs, temperate and tropical pastures, novel forages), plant products (plant extracts, essential oils, feed additives) and plant secondary compound fractions that have the potential to reduce methane from rumen microbes. However, the extent and the mechanism of action of these antimethanogenic effects are unknown. The aim of this project was to deliver information that could increase the likelihood of success in reducing methane emissions from the rumen and decreasing the ecological footprint of the livestock industries, and assist in the design of CFI methodology.

The results from recent studies have demonstrated that different classes of compounds may be responsible for the antimethanogenic effect in the rumen. For example, in the legume *Kennedia prorepens* the effect was linked to tannins (unpublished data), whereas in *E. glabra* the effects are likely to be linked to compounds other than tannins (Li et al. 2010). Furthermore, the antimethanogenic compounds in *E. glabra* seem to be from a different fraction to those responsible for other bioactive effects in the rumen, i.e. lactic acidosis (G. Flematti, pers. comm.). We also demonstrated that the profile of plant secondary compounds in *E. glabra* changes significantly when exposed to rumen microbes (G. Flematti, pers. comm.). It is not known if these plant x microbial metabolites are in fact the ones that are responsible for this antimethanogenic effect or not, as microbial conversions of secondary compounds from other plants and compounds are reported in the literature (Cox 1985, Hegarty *et al.* 1979).

The mechanism of the antimethanogenic effects in these plants and products remain largely unexplored. In collaboration with Dr Chris Mc Sweeney, we tested selected plants and plant extracts against a panel of rumen methanogens in pure culture and in mixed rumen culture. We found that plant extracts from *Eucalyptus occidentalis* and *E. glabra* can completely inhibit methane production by pure cultures of methanogens (C. McSweeney, pers. comm.). Our results also indicate that the effects could stem from a direct antimicrobial effect on methanogens and/or an indirect effect on hydrogen supply in the rumen. In these studies, we have also observed some dissociation between methanogen numbers and their activity, which is consistent with the literature (Zhou et al. 2011). This implies that other ways, for example reducing the metabolic activity of methanogens or stimulating microbes and other competitive metabolic pathways in the rumen may be occurring.

Microbial profiling techniques have evolved significantly in recent years to allow analysis of numerous environmental samples and detect fine changes in microbial populations. Dr McSweeney has developed protocols to examine shifts in rumen microbial populations. In our previous collaborative project we commenced molecular profiling of rumen populations as affected by *E. glabra* as a model antimethanogenic plant.

Our aim was to investigate the mechanisms of action behind the antimethanogenic bioactivity of the most significant bioactive plants and plant products at both the microbial ecology and cellular levels. The information generated in this project will assist in a better design of methodologies for reducing methane emissions from the rumen, but may also provide a biomarker for future screening endeavors.

9. Methodology

Overall design

We used Method 1 to isolate, examine, describe and provide plant chemical compounds for further testing.Method 2 and 3 helped to reveal those that were responsible for the antimethanogenic effect in a mixed rumen population, whileMethod 4 characterised the nature (mode of action) of their effects more specifically in pure cultures.

Method 1: Isolation and identification of bioactive compounds

Plant material was obtained from variety of sources - from an existing collection held at UWA, to those collected in-field (NLMP Projects BCCH.6540, BCCH.6520, B.CCH. 6410 and other projects outside NLMP and in collaboration with UWA, CSIRO, DEPI and DAFWA).

E. glabra (accession number SA 45599) was grown at the experimental site at the UWAFuture Farm, University of Western Australia (Pingelly, Western Australia) in winter 2011 (average monthly rainfall 24.1 mm. max temp 42.1℃, min temp 13.6℃, and mean temp 24.0℃). Leaves and stems <5cm long were harvested from approximately 1200 individual plantsat the post flowering stage (< 1 year old) in August 2011.Plant material was freeze-dried and kept in a sealed containerbag at room temperature until used for experimentation. Samples were ground through 1.0 mm screen (Glen Creston, Stanmore, England) and then used for extraction.

B. pelecinus cv. Casbah was obtained from a glasshouse experiment (Banik et al. 2013). Additional plant material was sourced in 2013 from UWA Shenton Park research station, in conjunction with UWA/DAFWA *in vivo* experiment, and used in the RUSITEC testing (B. CCH 6540) and subsequent HPLC profiling of RUSITEC fermentation fluid (this project). Finally, material was obtained from a glasshouse experiment (B. Banik, PhD experiment) in Aug 2013 to obtain enough material (fractions) for testing in pure culture. Extract from the chloroform/methanol solvent was selected for further work, as it had the highest activity amongst the five extracts tested (B. Banik, MSci thesis & Final Report B. CCH 1024).

Additional bioactive plants were sourced as follows: *Dorycnium hirstum* cv Canaritis and *Cichorium intybus* cv Choice from B. CCH 6540; *Leucaena leucocephala* and *Calliandra calothyrsus* from B. CCH 1012 (Durmic et al., 2012); and *Kennedia prorepens*from Enrich 1 plant collection (Durmic et al., 2010). Plants were extracted using various solvents and extracts were analysed in an *in vitro* batch fermentation system, as described further in the text. Several commercial products such as sesamin, essential oils, essential oil compounds and commercial grape tannins were obtained from external suppliers and details are listed accordingly in the experiments. Details on these are listed in corresponding experiments.

Plant extracts, compounds, fractions and pure compounds were obtained through collaborative links and through commercial suppliers, where required. The plant extraction and fractionation work was done in collaboration with Dr Gavin Flematti and postgraduate students under his supervision (School of Chemistry and Biochemistry, The University of Western Australia). Several types of fractionation procedures such as, thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and HPLC-Mass spectrometry (HPLC-MS) were used to fractionate the plant extract into smaller compounds and to detect, purify and help identify the major or most abundant compounds/fractions for testing. Plant material was first extracted with methanol/chloroform and fractions subjected to a bioassay-guided fractionation.

A follow up extraction was performed with methanol/chloroform extract for column chromatography. Extraction was done with 5 g of plant material in 150 mL of the solvent. The filtered extraction was evaporated under vacuum in preparation for separation. An alumina column equilibrated with hexane was loaded with the plant extract. This was separated using various systems from 100% hexane, through to 100% ethyl acetate, then to 100% methanol. The fractions were concentrated under vacuum, and dried under nitrogen. Extraction was carried out with 5 g of plant material in 150 mL of the solvent and stirred for 1 h. The mixture was filtered (Whatman No.1, 24 cm) and evaporated to dryness using a rotary evaporator. This was followed by silica gel fractionation and acid fractionation. Briefly, a column containing silica gel (Merck silica gel 60) was equilibrated with hexanes before loading with the plant extract dissolved in a minimum volume of methanol/chloroform. The column was then eluted with various solvents (100 mL each) from 100% hexanes followed by 100% hexanes

(×4), 20% EtOAc/hexanes (×2), 40% EtOAc/ hexanes, 60% EtOAc/ hexanes, 80% EtOAc/ hexanes, 100% EtOAc,20% MeOH/ EtOAc, 50% MeOH/ EtOAc and finally 100% MeOH. Fractions collected this way were concentrated under vacuum, and dried under a stream of N_2.

The most active fractions were tested in an *in vitro* batch fermentation system (IVFT, Method 2) and in pure culture (Method 4)asdescribed below. As the separation went further, the original plant amount was doubled or tripled to account for losses over the separations. In general, all fractions were dissolved in 500 µl of 70% ethanol (EtOH) for testing. This was to keep it consistent and in the right concentration range for activity andto secure 100 µl for each lab replicate.

Method 2: In vitro *fermentation of selected candidates in batch culture*

Plant extracts, fractions and pure compounds obtained by Method 1 were tested in a bioassay-guided fractionation. This was a step-wise process, coupled with Method 1, whereby fractions were tested in an *in vitro* batch system (IVFT) designed to test plant extracts (Durmic et al. 2008). Batch culture is a rapid and relatively inexpensive *in vitro* method to screen large numbers of samples for their effects on rumen fermentation and to identify plants and/or plant compounds with the potential to reduce methane production from fermentation. The batch culture system is a useful screening step prior to embarking on more expensive and time-consuming methods that are ultimately needed to provide more detailed information about the possible compounds and mechanisms involved in reducing methane emissions. Because it is using a full suite of microbes and relying on their interactions in a mixed microbial population system as it would occur in the animal, *in vitro* batch cultures can provide useful information on the general functionality of the rumen microbial system in response to plants and their products.

Briefly, fractions were tested in IVFT with oaten chaff or a concentrate diet as fermentation substrate to examine their antimethanogenic activity, and after each round of testing, the best fractions (in term of low methane production) were re-fractionated and retested in the system. The effects of the most active substrates on the functionality of the rumen microbial system were assessed by measuring common fermentation parameters including microbial gas production and composition (methane), and where applicable, volatile fatty acids (VFA) and NH_3 production.

Method 3: In vitro fermentation of selected candidates in RUSITEC

The significance of this method is that the RUSITEC is a continuous culture system and mimics a functioning rumen better than the batch culture system. Like batch culture, it is initiated using a mixed rumen population from donor animals, but it is an open system where substrate ('feed') is added daily, buffer ('saliva') is infused and end-products removed continuously over several weeks.

As such, it has several advantages. For example, the rumen microbial population need time to adapt and react to new environment. This means that over time, as a consequence of constant daily exposure, the effect may be intensified as microbes become more sensitive, or conversely,or diminish as the microbes adapt. The RUSITEC therefore enables us to examine the persistence of treatments in the rumen and at the same time permits samples to be taken to examine the microbial metabolites produced during fermentation of the compound and temporal effects on the ruminal microbial ecology.

In general, we followed a protocol of Li et al. (2013)designedfor testing antimethanogenic bioactive plant. Briefly, after period of acclimatization (7 days), selected candidates (plants or pure plant compounds) were tested over 15 days, with regular supplementation of fresh substrate and compound, buffer and removal of end-products. Samples were collected daily for assessment of functionality of the rumen microbial system (gas, methane and chemical profiling of microbial metabolites). Samples of the fermentation fluid were taken for chemical analysis to examine the metabolism of the compounds of interest by detecting their disappearance and formation of secondary metabolites post fermentation using a HPLC analysis. For this, ethanolic extracts of plant

material were prepared and filtered into HPLC vials. Fermentation liquid was collected from RUSITEC vessels with selected treatments at day 10 and day 20 and filtered. Extracts and fermentation fluid filtrates were subjected to HPLC analysis.

Samples from the most promising candidates/treatments were also submitted for molecular microbial analyses. The DNA from selected samples was extracted for quantitative real-time PCR (qPCR), using SensiFast^{TM} SYBR Lo-Rox (Bioline (Aust) Pty. Ltd). Primers for amplifying.*F. succinogenes, R. flavifaciens* and *R. albus* were the same as in Denman and McSweeney (2006) and for methanogens, the mcrA gene specific primer was used (Denman et al., 2007). In addition, specific 16S primers for RCC group (RCC1) and *Methanobrevibacter* spp. (Mbr) (both unpublished) were used to target these predominant ruminal methanogens. Real-time PCRs were run on ABI ViiA7^{TM} Real-time PCR machine using a standard 60^{o}C annealing with 40 cycle PCR and melt-curve cycle and results analysed using their proprietary software. Relative quantification of the target population was analysed by the cycle number at which an amplification plot crosses threshold fluorescence level (C_T). This C_T value can be directly correlated to the starting target concentration of the sample. The $2^{-\Delta\Delta CT}$ method was used here to calculate relative changes in gene expression determined from real-time quantitative PCR experiment. Using $2^{-\Delta\Delta CT}$ method for gene expression and microbial number counting was well documented by (Livak et al., 2001).

Genomic DNA (gDNA) was amplified by PCR using special primers targeting the 16S-V4 bacteria/archaeal sequences according to the protocol of Kozich et al., (2013) using the Illumina's MiSeq platform capable of producing 250-nucleotide paired end reads. This protocol used dual-index paired-end sequencing approach using 16S primers with a unique 8-nucleotide index or bar-code embedded in primers, each unique for every sample by a 8x2 (16) by 12x2 (24) array of unique bar-codes, capable of individually bar-coding 384 community samples.

PCR using proof-reading Platinum Hi-fi Taq Ploymerase was done as per the protocol for a maximum of 25 cycles using seta of forward and reverse bar-coded primers. Briefly, PCR set up (25 µl/sample) used the Platinum Taq-Hifi protocol to make master-mixes with 1 µl of 10 µM each of forward and reverse primers from an array for 1 µl each of the samples (~50 ng/µl). Cycling conditions (25 cycles) were as per Kozich et al (2013) protocol.

PCR products were run on a gel and quantified by Qubit assay (Life Technologies) and/or on-gel estimation of bands using Bio-rad Quantity software using DNA ladder as standards. Equimolar volumes of each product were calculated and pooled into a single tube. Pooled samples were concentrated to a total volume of 80-100 µl and gel purified on a 1.5% Agarose gel, the band cut out of the gel (stained by SYBR-safe) and gel-extracted using QIAQuick gel Extraction Kit (Qiagen) as per their protocol. The purified pooled bar-coded products were again run on a 1.5% gel to do a quality check and quantitate using the Qubit assay.

Where a strong antimethanogenic compound had specific effects on methanogens or relevant bacterial species, we examined its mode of action on the microbial species at the molecular (cellular) level using high volume next generation sequencing platforms and transcriptomic analyses to inform us about genes that are actively expressed and change in response to different substrates. Phylogenetic-based methods targeting the 16S rDNA gene were used to deep characterisation the bacterial and archaeal populations present in the fermentation samples by applying a high throughput sequencing platform and barcode 'pyrotagging'. Briefly, genomic DNA (gDNA) was obtained from the RUSITEC fermentation samples and amplified by PCR using special modified universal bacterial primers (515F and 806R) targeting the 16S-V4 bacteria/archaeal sequences according to the protocol of Kozich et al., (2013) using the Illumina's MiSeq platform capable of producing paired 250-nucleotide paired end reads. Specific sequences matching the Illumina Miseq sequencing adaptor P5 were added to the 515f primer, while the P7 adaptor was added to the 806r. This protocol, in addition, uses a dual index primer approach with index primers attached to both the forward and reverse primers, allowing for a multiplexing of 384 samples from 24 unique forward indexes and 16 reverse

indexes. Each individual DNA sample was amplified using a unique index combination sequencing approach with a unique 8-nucleotide index or bar-code embedded in primers, thus, as each is unique for a single sample, an array of 8x2 (16) by 12x2 (24) unique bar-codes, capable of individually bar-coding 384 community samples. The universal primers are capable of deep sequencing both total bacterial and archaeal kingdom targets and are ideal for use in microbial community analysis of 'prokaryotes'.

Short read sequence data generated using the Miseq platform was analysed using the QIIME: Quantitative Insights Into Microbial Ecology software package (Caporaso *et al.,* 2010), for generation of operational taxonomic units (OTU) clusters at a 97% similarity cut-off, alpha and beta diversity measures and distance calculations using Unifrac and further analysis using R with the Ade4, phyloseq (Thioulouse et al. 1997, McMurdie and Holmes 2013).

This relatively new sequencing protocol enabled a complete ecological analysis of the effect on all bacteria and methanogens in a fermentation system that modelled the rumen simultaneously and provides the best opportunity to predict the likely impact of antimethanogenic plants on the entire rumen microbial ecosystem.

Method 4: Testing the fractions and purified compounds in pure culture of methanogens.

We attempted to determine the mechanism(s) by which the most promising compounds or extracts identified in the other activities act on specific microbial populations to reduce the amount of methane produced. We tested the most potent plant fractions and selected pure compounds for their antimicrobial properties against key rumen methanogens grown in pure culture. We examined the extent of the effect by monitoring microbial cell growth and their activity (methane production) when exposed to the fractions/compounds. By having the most purified extracts that retain antimethanogenic properties and testing them in pure cultures, we were be able to confirm the nature of the action (broad, i.e affecting all types of methanogens) or specific (i.e. affecting only some types of methanoges).We also tested if the microbial cells recover after the removal of active fraction/compound, to distinguish between a simple static (cells regain activity in fresh media) or cidal (cells do not regain activity in fresh media) effect. We opted to progress the two diverse types of candidates i.e. fractions (mix of compounds) and a pure compound, for two reasons. We adopted an approach to test a broader mix of compounds, present in selected active fractions, as the plant secondary compounds(PSC) in selected plant (biserrula) are still unknown. Hence, we relied on representatives of the variety of PSCs in these fractions to maximise possibility of finding the active ones. We also wanted to examine if the mix of compounds would have a more diverse and persistent effect compared to a single compound.

Methanobrevibactergottschalkii, *Methanobrevibacterruminantium*, *Methanobacteriumbryantii*, *Methanosphaerastadtmanae*and one of the RCC group methanogens, *Methanoplasmagallocaecium* were selected as key rumen methanogens (Janssen et al., 2008) for pure culture analysis. The methanogens were grown in modified BRN media (Balch et al., 1979). Cultures were propagated under anaerobic conditions and headspace of H_2 and CO_2 (150 KPa H_2) at 39°C.

Methanogens were cultured in anaerobic BJ media which is a modified, sparse version of BRN (modified Balch media, Maczulack et al., 1989) media and uses only 10% of peptone and yeast extract of the original recipe and lacks ammonium chloride, sodium acetate and sodium formate. Briefly, the constituents are prepared anaerobically (per litre) with 50 ml each Mineral solution #2 & #3, 1ml each of Pfenning trace element solution and 0.1% Resazurin (see McSweeney et al., 2005), 100 ml clarified rumen fluid, 0.25g each of yeast extract and Peptone, 6.0g of sodium hydrogen carbonate and 0.4g of cysteine HCl, all topped up to 1L with anoxic water (boiled) and rendered anaerobic by sparging with CO_2. The pH was adjusted between 6.8-7.0 and 10 ml each was dispensed into Balch tubes (Bellco Glass, Vineyard, NJ) inside a Coy Anaerobic chamber, closed with

blue butyl stoppers and sealed with aluminium crimp caps (Wheaton Industries Inc., Millville, NJ). Tubes were autoclaved at 121^{o}C under 15 psi for 20 min (Balch et al., 1979).

Methanogen strains were revived from glycerol stocks and transferred at least twice in BJ media under hydrogen, and grown with their respective substrates. When the cultures reached early exponential phase (different for each methanogen), between OD_{600} 0.15- 0.5, measured on a Spectronic 200 (Thermo Scientific, Australia), their numbers were determined using a Thoma Counting Chamber (x1000 magnification). The enumeration of the methanogen cultures prior to the start of testing the fractions was necessary to ensure that the methanogen numbers were similar in proportion to their numbers in the rumen fluid. It is reported that there about ~ 10^{10} -10^{11} bacteria/g of rumen fluid/digesta (Hobson, 1997 and Mackie, 1997); of these, methanogens are approximately 0.1% (~10^{7}-10^{8} cells/g). Therefore, the effect of essential oil compounds on rumen methanogens in the IVFT assay was likely to be inhibitory on ~10^{8}-10^{9}methanogens /10 ml of rumen fluid. The rationale that the ratio of the number of methanogens to the concentration of essential oil compounds in pure culture tests should represent approximately a similar ratio used in the IVFT experiment was the reason to test them under these conditions.

In an anaerobic hood, these enumerated methanogen cultures were inoculated at 1:100 into fresh, sterile pre-warmed 250 ml BJ media giving final concentrations of ~ x 10^{8} cells/ml after thorough mixing. This media contained supplemental methanol and TMA if required. In a sequential manner, 10 ml of diluted cells and media were added to quadruplicate individual pre-warmed balch tubes. Next 6.3 □l of each of the essential oil or essential oil compound was dispensed in the same way.

All cultures were done in quadruplicate. The initial OD at 600 nm was recorded and thereafter at appropriate intervals until 72h. Gas pressures were recorded immediately after measurement of optical density. For this, a custom-made Luer-lock, disposable needle-holder was attached to a Honeywell pressure transducer (Honeywell Intl. Inc.), which in turn was connected to a Vellman data-logger (Vellman Instruments, Belgium). The data-logger records voltages displayed on the Vellman PC-Lab 2000 software. These voltages were then converted to kPa by a pressure-voltage calibrated formula.

In EO/EOC testing, initial tests were carried with two methanogens at a concentration of 2 µl/ml of media, which corresponded with the lowest dose that showed reliable effects for those tested in RUSITEC experiments. Based on these preliminary experiments that caused a complete inhibition across all compounds, this was modified to add only 0.625 µl/ml of media. This was the lowest dose tested in RUSITEC experiments. A final round included a dose response experiments with a smaller subset of methanogens representing three major genera against the two compounds that showed significant effect at this lower concentration, as well as two others that were shown to be effective at higher doses in RUSITEC trials. Similarly, four compounds were tested further in dose response experiments at appropriate concentrations. To minimise pipetting error of dispensing very small volumes of oily compounds, they were dissolved in a non-toxic quantity of anaerobic ethanol and a larger volume of anaerobic diluent. Control tubes contained identical concentrations of such ethanol and diluent.

Biserrula fractions dissolved in ethanol were added to a pure culture of methanogens in log phase (i.e. cell count 1.0 – 3.0 x 10^{7} cells/ mL). A culturegrown in the presence of 100 µL 70% EtOH were used as a control. All treatments were run in triplicate. Headspace gas pressure and methane concentration were measured before adding treatments and after 48h of incubation,when the culture reached late log phase. Visible growth was recorded and methane in headspace was measured by using gaschromatography (GC).

At the end of the incubation, an aliquot (1 – 2 mL) of culture was collected into QSP or Sarstedt 2 ml screw-capped tubes and immediately processed for microbial numbers by 16SrDNA. The DNA was extracted using a modified method of Denman and McSweeney (2006). Briefly, the genomic DNA was

extracted using a bead-beating method with Zirconium beads (1:1 (w/w) mix of 1mm+0.1 mm; ~ 200 – 250 mg) and the Fast Prep Instrument (Q-BIO gene, Quebec, Canada). Primers designed for the detection of methanogenic Archaea were targeted against the methyl coenzyme-M reductase (mcrA) gene (Tatsuoka et al., 2004; Denman et al., 2007). The mcrA forward primer (qmcrA-F) 50-TTCGGTGGATCDCARAGRGC was designed to target the conserved amino acid sequence FGGSQR while the reverse primer (qmcra-R) 50-GBARGTCGWAWCCGTAGAATCC targeted the GFYGYDL conserved amino acid sequence (Denman et al., 2007). The qRT-PCR assays were performed on a modified methods described in Denman and McSweeney, 2006 and Danmen et al., 2007, and use an ABI PRISM 7900HT Sequence Detection System (Applied Biosystem, USA). Assays were set up using the SensiFast SYBR Green qPCR Lo-ROX Mix (Invitrogen). The assays were set up using the reaction mixture (35 µL) included SensiFast SYBR Green qPCR Lo-ROX Mix (16.5 µL), the primers (both forward and reverse, 1.32 µL each), distilled water (12.86 µL) and gDNA template (1.4 µL) with the following cycle conditions: one cycle of 50°C for 2 min and 95°C for 2 min for initial denaturation, 40 cycles at 95°C for 15 sec and 60°C for 1 min for primer annealing and product elongation and further one cycle of 95°C for 2 min, 60°C for 15 sec and 95°C for dissociation analysis of melt curve (Denman et al., 2007). All methanogenic archaeal DNA from sampling time points was diluted 1 : 10 before use in quantitative real-time PCR.The treatments were compared with the control culture and $2^{-\Delta\Delta CT}$ fold differences of these treatments were calculated by comparing with the fold inhibition or fold increment with the control.
Gas pressure and methane measurements taken as appropriate at one mid-exponential phase time point (typically ~44 h) and endpoint (>72 h).Gas samples were also collected at the time of addition of the extracts and at the final time point in order to monitor H_2, CH_4& CO_2 levels by gas chromatography (GC). Gases were analysed on a Shimadzu 2014 GC using a thermal conductivity detector (TCD). Gases were separated by a 1.8 m Haysep-Q packed column (2.0 mm ID) at 38^{o}C (Injector at 75^{o}C and detector at 100^{o}C) with N_2 as carrier gas with a total flow of 25.0 ml/min. Results were quantified by Shimadzu LabSolutions software using 5-levels of gas-mixture standards containing H_2, and CH_4 in nitrogen. Methane production was calculated as µmol/10 ml of culture with a headspace of ~17 ml.

Methanogen cultures showing reduced growth and inhibition of methane production by the biserrula fractions (compared to the ethanol controls) were tested for the static or cidal action of the compounds by inoculating those cultures into new BJ media. An aliquot of 100 µl / 10 ml from these inhibited cultures were inoculated into fresh media with the requisite substrates and hydrogen, but without any added compounds. Growth (OD_{600}) and pressure measurement were recorded at the beginning and after 72h as before and methane production estimated by GC analysis (data not shown). A static effect was defined as the production of methane along with growth (OD_{600} increase) by the subculture from an inhibitory essential oil compound. A cidal effect was defined when no growth nor methane production was measurable in the subcultures from an inhibitory compound.

To examine the mechanism of the effect (static vs cidal), an aliquot (200 µL) of overnight culture that was already treated with an active fraction was inoculated into a blank media. The culture was grown in the same environment as described previously and methane was measured at the end of the incubation. Methano-cidal effect was defined if no visible microbial growth occurred and no methane production was detected; equivocal methano-cidal effect was considered if there was some microbial growth and methane production of < 70 µmol; and methano-static effect was if there was > 70 µmol methane detected in headspace.

10. Results

The results for thisfinal report have been divided into sections, covering separate components of the work:

1. Isolation, identification and *in vitro* testing of fractions from *Eremophila glabra*

2. Isolation, identification and *in vitro* testing of fractions from *Biserrula pelecinus*

3. *In vitro* testing of essential oils and essential oil compounds

4. Testing of fractions and pure compounds against methanogens in pure culture

5. *In vitro* testing of extracts and fractions from other bioactive plants

6. *In vitro* testing of grapemarc

1. Isolation, identification and *in vitro* testing of fractions from *Eremophila glabra*

Overview

In this part of the project, we aimed to determine the active fractions of *E. glabra* that would be responsible for low methanogensis. A total of 3 rounds of fractionations were completed, with several active fractions obtained and one pure compound (i.e. sesamin) identified.

Background

In our previous work, we identified*E.glabra*as a potent antimethanogenic plant when fermented *in vitro* by rumen microbes (Durmic et al., 2010). Further work had confirmed that extractable compounds in this plant are responsible for the effect (Durmic et al., 2013) and may inhibit methanogens in pure culture (B. CCH 1012 Final Report). Tannins are often identified as main phytochemicals responsible for low methanogenesis(Hess et al., 2003; Hart et al., 2008; Kamra et al., 2008; Patra et al., 2010), however in this plant tannins have been ruled out, as the activity was maintained after the addition of a tannin binder (B. CCH 1012 Final Report).

Further investigation into the activity of this plant revealed that bioactive compounds can be isolated using different solvents (except water), and that the most potent crude extract was one obtained using methanol/chloroform. Initial testing of crude plant extractions demonstrated that methanol/chloroform (1:1, v/v) had the most potent activity amongst the crude extracts of *E. glabra* (B. CCH 1012 Final Report). The HPLC analysisalso showed that this extract contained the most diverse spread of compounds (G. Flematti, pers. comm.).

Experiment BA 6 - first fractionation of *E. glabra*

Aim: to isolate and test in IVFT fractions from methanol/chloroform extract of *E. glabra*

Results and discussion

A total of 12 fractions were obtained and amongst these, there were five that significantly reduced methane when compared to the control (Figure 1.1). The most potent fraction was fraction 4 that produced ten times less methane than the control. This fraction was the only one that caused small (about 15%) but significant reduction in overall microbial gas (Table 1.1). Other fractions caused 20-80% reduction in methane compared to control.

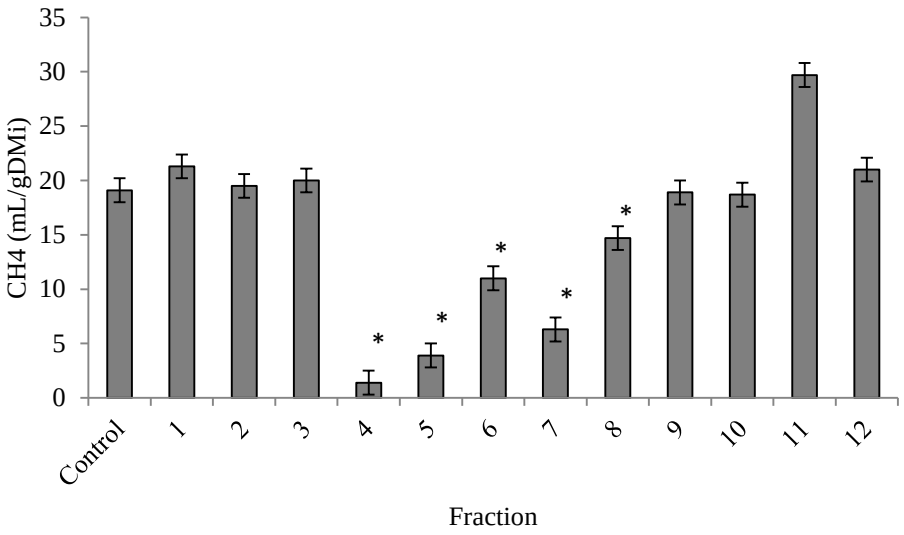


Figure 1.1. Methane production (mean ± SEM) by rumen microbes when fermenting oaten chaff in presence of selected fractions *in vitro.* * significantly (P<0.05) lower than the control. DMi - dry matter incubated. Control - oaten chaff + EtOH

Table 1.1. Gas production (mean ± SEM) by rumen microbes when fermenting oaten chaff in presence of selected fractions *in vitro.* * significantly (P<0.05) lower than the control. DMi - dry matter incubated. Control - oaten chaff + EtOH

Fraction	Gas production (mL/g DMi)
Control	354
1	358
2	360
3	358
4	302*
5	344
6	362
7	377
8	356
9	355
10	365
11	367
12	355
S.E.M.	2.7

Fraction 4 was selected for further examination as the most potent fraction. Reduction in gas was negligible and could be attributed to reduction in methane portion.

Experiment BA 10 - second fractionation of *E. glabra*

Aim: to derive fractions from fraction 4 and test using the IVFT

Results and discussion

Fraction 4 was subjected to silica gel fractionation to derive 33 fractions. These were analysed by TLC and combined into 8 fractions based on their similarity in TLC profiles. Each of these contained about 3-4 fractions as part of the 33 derived above, but the 33 were also kept separate so they could be tested individually if required. Fractions were tested in the IFVT.

There were six fractions that significantly ($P < 0.05$) reduced methane production when compared to the positive control. The level of inhibition varied, with fraction C4 producing 70% less methane than the control. All fractions that inhibited methane caused significant reduction in gas and VFA and only one reduced NH_3, however reduction in gas did not exceed 33% (C4), but reduction in VFA was up to 47% (with C4, Table 1.2). There was a strong correlation between methane and gas ($R^2 = 0.94$) or VFA ($R^2 = 0.93$), indicative of general antimicrobial effects. Since there were no fractions with obvious selective effect on methane, fraction C4 was selected for further testing as being the most potent fraction in reducing methane and overall fermentation.

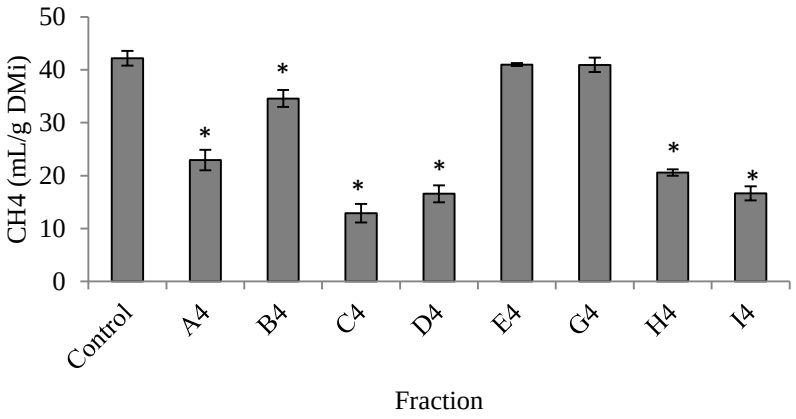


Figure 1.2. Methane production (mean ± SEM) by rumen microbes when fermenting oaten chaff in presence of selected fractions *in vitro.* * significantly ($P<0.05$) lower than the control. DMi - dry matter incubated. Control - oaten chaff + EtOH

Table 1.2. Fermentation products (mean) when oaten chaff was fermented by rumen microbes in the presence of selected bioactive fractions *in vitro.* * significantly (P<0.05) lower than the control. DMi - dry matter incubated. Control - oaten chaff + EtOH

Treatment	Gas (mL/g DMi)		VFA (mmol/L)		A:P	NH_3 (mg/L)	
Control	369		117		3.0	79	
A4	280	*	74	*	3.5	135	
B4	315	*	100	*	3.8	155	
C4	249	*	62	*	3.4	130	
D4	275	*	72	*	3.4	128	
E4	369		137		3.2	62	*
G4	375		122		3.0	75	
H4	281	*	72	*	3.3	130	
I4	273	*	67	*	3.4	127	

Amongst the fractions derived from fraction 4, three (C4, D4, I4) were selected for further testing, as being the most effective in reducing methane production.

Further HPLC profiling of four C4 fractions revealed that three of these, namely EG4-9, EG4-10 and EG4-11 were identical, and a single compound (Figure 1.3). The compound was identified as sesamin (5,5'-(1S,3aR,4S,6aR)- (tetrahydro-1*H*,3*H*-furo[3,4-c]furan-1,4-diyl) bis -1,3-benzodioxole (Figure 1.4).

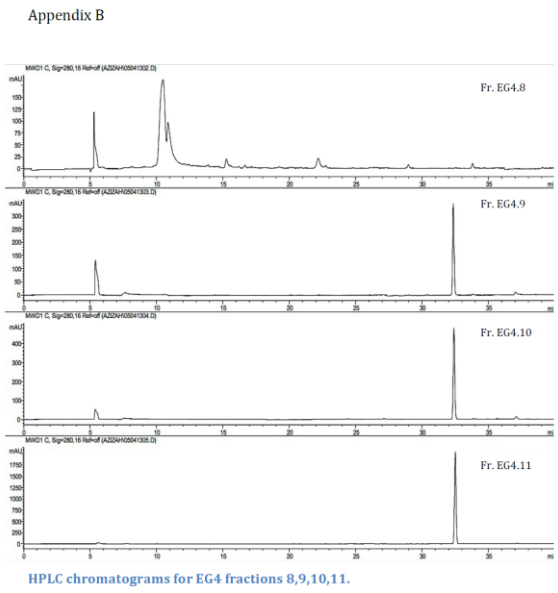


Figure 1.3. HPLC profiles of four fractions derived from fraction C4 (AzizahAlgreiby, MSci thesis)

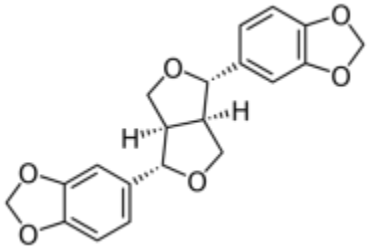


Figure 1.4. Structural formula of sesamin

HPLC of fractions derived from D4 and F4 revealed that contained a mixture of compounds (Figure 1.5)

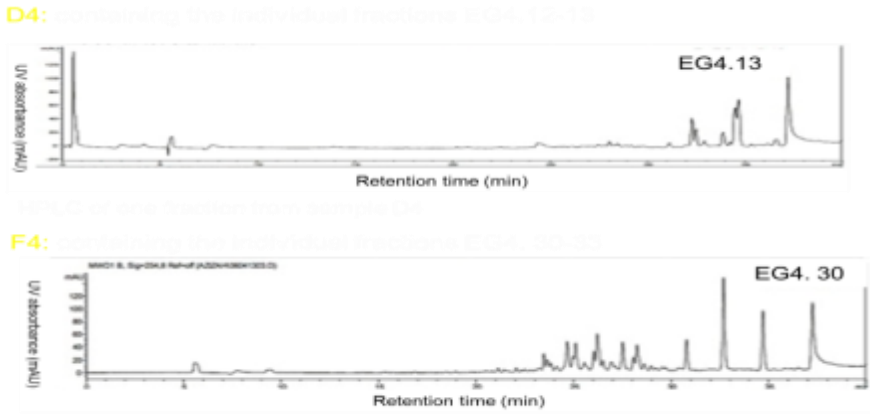


Figure 1.5. HPLC profiles of *E. glabra* fractions derived from D4 (EG4.13) and F4 (EG4.30) (AzizahAlgreiby, MSci thesis)

Three fractions from *E. glabra* showed antimethanogenic properties. In one of them, i.e. C4 an identical pure compound was detected in three sub-fractions and identified as sesamin.

Experiment BA 18 - third fractionation of*E. glabra*

Aim: to test in IVFT fractions derived from fractions C4, D4 and I4

Results and Discussion

Fractions were derived from C4, D4 and I4 (n= 3, 2 and 4, respectively, 9 in total), but only 8 fractions were tested, as there was not enough of EG4-11 to complete the testing.

The fraction EG4-10 had significantly reduced methane compared to control, with the reduction was around 40% (Figure 1.6). This fraction did not cause significant reduction in gas production when compared to control (Table 1.3).

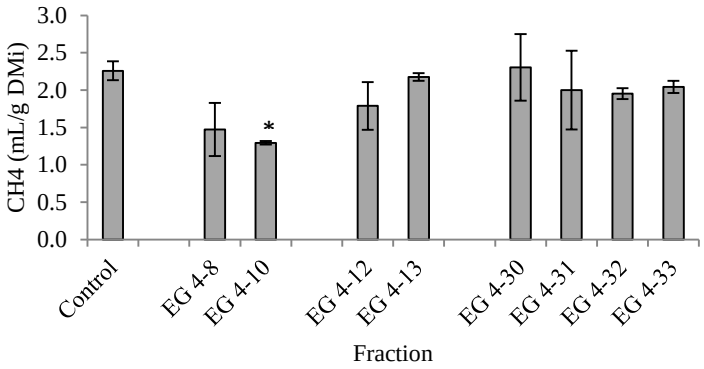


Figure 1.6. Methane production (mean ± SEM) by rumen microbes when fermenting oaten chaff in presence of selected fractions *in vitro.* * significantly (P<0.05) lower than the control. DMi - dry matter incubated. Control - oaten chaff + EtOH

Table 1.3. Gas production (mean ± SEM) by rumen microbes when fermenting oaten chaff in presence of selected fractions *in vitro.* * significantly (P<0.05) lower than the control. DMi - dry matter incubated. Control - oaten chaff + EtOH

Treatment	Gas production (mL/g DMi)	
Control	364	
EG 4-8	362	
EG 4-10	366	
EG 4-12	367	
EG 4-13	367	
EG 4-30	366	
EG 4-31	355	*
EG 4-32	364	
EG 4-33	369	
S.E.M.	3.0	

Fraction EG4-10 had pure compound and identified as sesamin which was decided as candidate for further testing. As a commercial sesamin is available, further testing was done with a commercial compound rather than reisolating compound from plant material.

Experiment BA 20/21

Aim: to test a commercially-available sesamin for antimethanogenic effect in the IVFT

Materials and methods

One mg of sesamin (Sesamin ≥95%, crystalline, S9314, Sigma Aldrich)was dissolved first in 1 mL of 70% EtOH (Sesamin S + EtOH) before 100 µL of this solution was included in each tube. Final concentration was therefore 0.1 mg/100 mg substrate. A control was oaten chaff + 100µL EtOH (Control + EtOH).

Results and Discussion

When sesamin S was incubated with oaten chaff, there was no significant reduction in methane or gas (Table 1.4).

Table 1.4. Gas and methane production (mean ± SEM) by rumen microbes when fermenting oaten chaff in presence of of sesamin S *in vitro.** significantly (P<0.05) lower than the control. DMi - dry matter incubated. Control - oaten chaff +EtOH

Treatment	Gas		CH4	
	(mL/g DMi)	SEM	(mL/g DMi)	SEM
Control + EtOH	389	2.6	47.8	0.5
Sesamin S + EtOH	385	2.0	47.3	0.4

This sesamin had poor solubility and did not dissolve well in 70% EtOH. It was recommended to try to dissolve this compound in DMSO to try to improve its solubility.

Experiment BA 23 - Commercial sesamin (Sigma) in different solvents

Aim: to examine if using different solvents may improve effectiveness of commercially-available sesamin.

Materials and methods

Commercial sesamin (Sesamin ≥95%, crystalline, S9314, Sigma Aldrich) was dissolved in either 70% EtOH (Sesamin S + EtOH) or DMSO (Sesamin S + DMSO) and 100µL was included per tube (100 mg oaten chaff substrate). The controls were oaten chaff + 100µL EtOH (Control + EtOH) or DMSO (Control + DMSO).

Results and Discussion

There was no reduction in methane in any of the treatments containing sesamin S (Table 1.5).

Table 1.5. Gas and methane production in the IVFT testing of sesamin S using different solvents. Significance: within column, values not sharing the same superscript differ (P<0.05). DMi - dry matter incubated. Control - oaten chaff

Treatment	Gas (mL/g DMi)		CH_4 (mL/g DMi)	
Control	407	d	55	c
Control + EtOH	426	a	69	c
Sesamin S + EtOH	428	a	73	a
Control + DMSO	415	bd	50	b
Sesamin S + DMSO	409	bd	51	b

There was still a problem of poor solubility of material and there was no reduction in methane when sesamin S was dissolved in either 70% EtOH or 10% DMSO. It was suggested to improve solubility by dissolving it in 100% first, before adjusting the EtOH concentration to 70% (G. Flematti, pers. comm.).

Experiment BA 24a - Sesamin S and modified EtOH (m EtOH)

Aim: to test sesamin S using modified 70% ethanol (m EtOH)

Materials and methods

Sesamin S was first dissolved in 700 µL of 100% EtOH, before 300 µL of water was added (Sesamin S +m EtOH). Following this, 100 µL of the mix was dispensed in each tube. Control was oaten chaff + 10 µL 70% EtOH (Control + EtOH).

Results and discussion

There was some, very small, but significant reduction in methane when sesamin S was dissolved by a modified 70% EtOH (Table 1.6).

Table 1.6. Gas and methane production in the IVFT testing of sesamin S dissolved in modified 70% EtOH. Significance: within column, values not sharing the same superscript differ (P<0.05). DMi - dry matter incubated. Control - oaten chaff + mEtOH

Treatment	Gas (mL/g DMi)		CH4 (mL/g DMi)	
Control + mEtOH	421	a	68	a
Sesamin S + mEtOH	412	b	65	b

Next step may involve increasing the dose of sesamin to increase these differences.

Experiment BA 26 - Commercial sesamin at higher concentrations

Aim: to test commercial sesamin from Sigma at higher concentrations and use mEtOH to improve its solubility.

Materials and methods

A commercial sesamin (Sesamin ≥95%, crystalline, S9314, Sigma Aldrich) was tested at level of 4 mg and 10 mg/g substrate. Sesamin S was first dissolved in 100 % EtOH, then appropriate amount of water was added to dilute EtOH to 70%. I.e. 10 mg sesamin was dissolved in 0.7 mL 100% EtOH and 0.3 mL of water was added, resulting in a stock solution of 10 mg/mL. Then 0.1 mL of this was added to each tube, resulting in 10 mg/g substrate.

Results

There was no antimethanogenic effect with commercial sesamin S at any of the doses tested (Table 1.7).

Table 1.7. Gas and methane production from sesamin S incubated with oaten chaff. Control - oaten chaff + mEtOH, AMC - antimethanogenic control (tea tree oil).DMi - dry matter incubated. * - significantly lower than the control

Treatment	Gas (mL/g DMi)		CH_4 (mL/g DMi)	
Control	388		43	
AMC	279	*	8	*
Sesamin S 4 mg/g	388		42	
Sesamin S 10 mg/g	397		44	

Discussion

It is suggested to i) test sesamin with different types of diet and ii) increase concentration further

Experiment BA 30b *In vitro* testing of commercial sesamin (Sigma) dissolved in ethanol with 2 different substrates

Aim: to examine if high nutritive substrate may improve effectiveness of low level sesamin.

Materials and methods

Commercial sesamin (Sesamin ≥95%, crystalline, S9314, Sigma Aldrich) was dissolved in 70% EtOH (sesamin EtOH) at level of 4 mg and 10 mg/ g of substrate (100 mg substrate per tube). In each tube, 100µL of the sesamin in EtOH was added to 2 different substrates, either forage (oaten chaff) or high-protein concentrate-based diet (EasyOne, Milne Feeds, Welshpool, Australia; 14.5% CP; ME 11.0 MJ/kg; crude fibre 20.0%).

Results

There was no reduction with any of the addition to any of the two diets (Table 1.8).

Table 1.8. Gas and methane production from sesamin S incubated with oaten chaff or concentrate diet. DMi - dry matter incubated.

Treatment	Gas (mL/g DMi)	CH4 (mL/g DMi)
Concentrate diet + EtOH	401	60
Sesamin S 4 mg + concentrate diet	399	61
Sesamin S 10 mg + concentrate diet	396	60
Oaten chaff + EtOH	383	52
Sesamin S 4 mg + oaten chaff	381	52
Sesamin S 10 mg + oaten chaff	382	53

Experiment 27b - *In vitro* testing of commercial sesamin (Bulk Nutrients)

Aim: to examine another commercial sesamin extract at higher inclusion levels.

Materials and methods

Sesamin extract (100%, Bulk Nutrients, Grove, Tas, Australia, 'Sesamin BN') was included at level of 100 mg, 500 mg and 1000 mg per g of substrate (oaten chaff). Extract was added directly to the tube, without dissolving in ethanol.

Results

The addition of sesamin BN at the 100 mg/mL dose resulted in a significant decrease in methane (Table 1.9). The gas production was also inhibited with all except 1000 mg/g.

Table 1.9. Gas and methane production from commercial sesamin BN incubated with oaten chaff. PC - substrate control (oaten chaff). Control - oaten chaff. DMi - dry matter incubated. * - significantly lower than the control

Treatment	Gas (mL/g DMi)		CH_4 (mL/g DMi)	
Control	414		53	
Sesamin BN 100 mg/g	313	*	42	*
Sesamin BN 500 mg/g	409	*	54	
Sesamin BN 1000 mg/g	467		54	

Discussion

It appears that we have found the dose and the form of sesamin that can potentially inhibit methane by 20%, but this effect was accompanied with a reduction in overall gas. There needs to be more testing to determine if the lower doses (i.e. those between 10 mg and 100 mg/g) may have less effect on gas production. Solubility of the compound is also an issue. It seems that there was some effect when the compound was added directly to the system, without using a solvent.

Another aspect to look at is the detailed chemical configuration of sesamin. In discussion with UWA Chemistry, we learned that sesamin contains 4 stereogenic carbon atoms, which means it can potentially exist in 16 possible configurations or stereoisomers. (+)-Sesamin and epi-sesamin are major lignans in refined sesame oil. However, epi-sesamin is rarely found in nature, but rather generated during processing (roasting, bleaching). It is derived from sesame seeds and commercially available sesamin supplements usually contain both (+)- sesamin and episesamin at an approximate 1:1 ratio(Li et al. 2005). Further, studies have found that enantiomeric ratio of sesamin to its enantiomer in the bark of a bioactive plant *Pentaspadon motley* (Anacardiaceae) is 1:0.86 (Kitamura et al. 2011). Investigation on these differences in structure between Sigma/*E. glabra*/Nutri sesamin is planned and may provide an explanation why analogues of sesamin are not inhibitory to methane. It is also possible that some other compounds present in the original fraction 4 are required for the activity of sesamin.

This was the last and concluding experiment on *E. glabra* bioactivity under NLMP funding, but the work continues as part of PhD project at UWA.

General discussion

This study confirmed that *E. glabra* contains fractions with anti-methanogenic activity, with selected fractions of *E. glabra* capable of reducing methane production by up to 90% when compared to control. We have identified one purified compound from *E. glabra*, sesamin, but we could not confirm

unequivocally its capacity to reduce methane from rumen microbes. Sesamin is a lignan commonly found in some plants and has been reported to have many bioactive properties, including antioxidant, anti-carcinogen, and anti-hypertensive activities, and are capable of reducing serum lipids (Trattner et al., 2008; Lee et al., 2011; Trattner et al., 2011). It has potent antibacterial properties against gut microbes (Zhou et al., 2004). In fish, sesamin increases conversion of linolenic acid to docosahexaenoic acid (Trattner et al., 2008), and the latter is found to inhibit methane production by rumen microbes (Fievez et al., 2007). Further experiments may focus on determining an appropriate source, dose, solvent type and other conditions, as well as rule out any toxic effect on rumen microbes in mixed and in pure cultures.

The reduction in total gas and VFA (where tested) with all fractions that were antimethanogenic demonstrated that fractions of *E. glabra* may have a more general effect on rumen microbes, which is consistent with fermenting *E. glabra* plant (Durmic et al., 2010) and activity towards bacterial populations in the rumen (Li et al. 2013). It should be considered that the level of reduction in methane was always greater than the level of reduction in gas, which implies there was some specific effect on methanogens. However, the potent effect of some fractions on VFA production implies that there was some severe inhibition of rumen fermentation, so care should be taken and appropriate doses determined that have less detrimental effect. In our previous studies, we have demonstrated that it is possible to dilute out negative effects of *E. glabra* on rumen fermentation, while still maintaining the antimethanogenic effect *in vitro*(Li et al., 2013) and *in vivo* (B. CCH 1012 Final Report).

Conclusions

Extractable compounds of *E. glabra* are responsible for the antimethanogenic effect in this plant. Sesamin was the first purified compound identified in an antimethanogenic fraction from this plant, but we could not confirm its potential antimethanogenic effect unequivocally when tested from commercial sources. A variety of other bioactive fractions from *E. glabra* were also isolated, offering a range of pathways for further investigation towards developing potent methane inhibitor. Identifying active fractions from *E. glabra* may also help us target plants that contain similar compounds and therefore discover other antimethanogenic plants without the need to go through the whole process. It should be emphasised that antimethanogenic activity in some fractions disappeared after multiple separation steps were undertaken and it seems that there is a combined effect among fractions of *E. glabra* that contribute to low methanogenesis. Some antimethanogenic *E. glabra* fractions affected microbial fermentation and require further investigation into determining an appropriate dose.

Implications

The studies confirmed that extractable compounds from *E. glabra* can reduce methane production, however, the full suite of pure compounds responsible for this effect have not yet been isolated and activity confirmed. Sesamin may have a role in the antimethanogenic potential of *E. glabra*, but the exact structure and conditions need to be carefully investigated and confirmed before any advancements towards *in vivo* can be made.

2. Isolation, identification and *in vitro* testing of fractions from *Biserrula pelecinus*

Overview

The aim of this part of the project was to examine the bioactivity of biserrula in more detail, including effects of crude extracts and fractions, on rumen fermentation and methanogens *in vitro*.A total of 3 rounds of fractionations were completed, with several active fractions obtained and tested in the IVFT (Method 2) and in pure culture (Method 4). In addition, fermentation liquid samples were obtained from a RUSITEC study in B.CCH 6540 and analysed by HPLC for changes of plant compounds when

fermented by rumen microbes.

Background

A preliminary study of some common pasture forages in Australia revealed that *B. pelecinus* (*Astralagus pelecinus*, biserrula), when fermented by rumen microbes, produced nearly ten times less methane than the other legumes tested, such as *Trifolium subterraneum* L. and *Medicago sativa* L. (Banik et al. 2013a,b). While it reduces overall gas production, it sustains VFA production by the rumen microbes and produces some other favourable fermentation profiles, such as enhanced propionate. The mechanism behind low methanogenic potential in biserrula is unknown, and while proanthocyanidins are found in other *Astragalus* species (Aerts *et al*. 1999), in biserrula, the type, amounts, distribution of PSC or their effects on rumen fermentation are unknown.

Further, in our previous studies (B.CCH 1012) we observed that a bioactive plant fermented by rumen microbes in the RUSITEC produced different HPLC profile than the plant itself. We have also documented that while some peaks are disappearing, others are appearing. This triggered interest in the metabolites in antimethanogenic plants for two reasons - a) if the bioactive PSC are degraded by rumen microbes, then the activity may be reduced; b) if novel peaks (compounds) are appearing as microbial metabolites, while the bioactivity remains, those newly formed ones may be in fact be responsible for the antimethanogenic effect.

Finally, fractions obtained from biserrula provided ideal candidates to enhance our knowledge and understanding of antimethanogenic activity of fractions in pure cultures. In addition to testing some pure essential oil compounds, we have also tested a mix of compounds (i.e. fractions) from biserrula and compared these findings to those obtained by using a single compound.

Materials and methods

In general, extraction and separation of fractions were conducted as described in Method 1, IVFT were conducted as per Method 2, followed by a pure culture testing (Method 4) of the most active fractions. In addition, fermentation liquids were obtained from the RUSITEC experiment conducted in B.CCH6540 (ELLE) and analysed by HPLC to examine degradation of plant compounds and formation of secondary metabolites in the system (Method 3).

Experiment BA 4a - First fractionation of *B. pelecinus*

Aim: to isolate and test in IVFT fractions from methanol/chloroform extract of *B. pelecinus*

Results and discussion

Chloroform/methanol solvent was used to obtain the initial crude extract from *B. pelecinus*. This extract was then fractionated using silica gel and acid fractionation, giving 10 and 3 fractions respectively. These fractions were tested *in vitro* and their antimethanogenic properties are shown in Figure 2.1. Fraction numbers 1, 2b, 3 and 8 were most active.

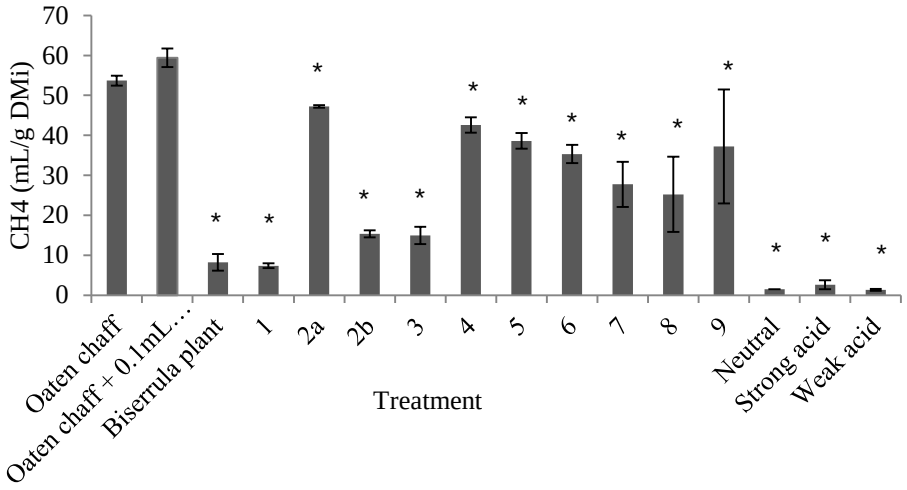


Figure 2.1: Methane production (mean ± SEM) by rumen microbes when fermenting oaten chaff in presence of selected fractions *in vitro.* * significantly (P<0.05) lower than the control. DMi - dry matter incubated. Control - oaten chaff (biserrula plant) or oaten chaff + EtOH (biserrula fractions)

The results for overall fermentation characteristics from the *in vitro* fermentation testing are provided in Table 2.1. Fraction 2b did not reduce gas and VFA; 3 caused a reduction in VFA and 8 caused reduction in gas. Ammonia levels were not reduced in any of the fractions. Fraction 1 was found to contain saturated hydrocarbons and was not selected for further testing. Fractions 2b, 3 and 8 were selected for further testing because they were the most active, and/or because they were not hydrocarbons and/or because they did not inhibit gas, VFA or both. While the acid and neutral fractions were all active, since there were no differences between these, they were not selected for further testing. While this process may simplify extraction, it is only useful when fractions differ in activity.

Table 2.1. Fermentation parameters by rumen microbes when fermenting oaten chaff in presence of selected fractions *in vitro.* * significantly (P<0.05) lower than the control. DMi - dry matter incubated. Control - oaten chaff + EtOH

Fraction	Gas (kPa)		VFA (mmol/L)		NH_3 (mg/L)
Control	100		133		142
1	110		129		135
2a	95		112	*	160
2b	103		129		143
3	105		119	*	140
4	90	*	128		154
5	88	*	118	*	157
6	85	*	112	*	146
7	88	*	122		190
8	62	*	121		250
9	87	*	120	*	170
Neutral	111		109	*	147
Strong acid	109		108	*	144
Weak acid	108		114	*	138

Experiment BA 11 - Second fractionation of *B. pelecinus*

Aim: Fractionate further fractions 2b, 3 and 8 and test in the IVFT

Fractions 2b and 3 showed similarity in compounds and were combined to derive 11 fractions (labelled A3 -K3), while fraction 8 was fractionated to 7 fractions (labelled A8-G8), resulting in a total of 18 fractions for testing (Figure 2.2).

Results and Discussion

Amongst the 18 fractions tested, 10 had significantly reduced methane (Figure 4), with five fractions having methane below 20 mL/g DM (i.e. less than half of the control), namely D3, F3, H3 and A8, E8.

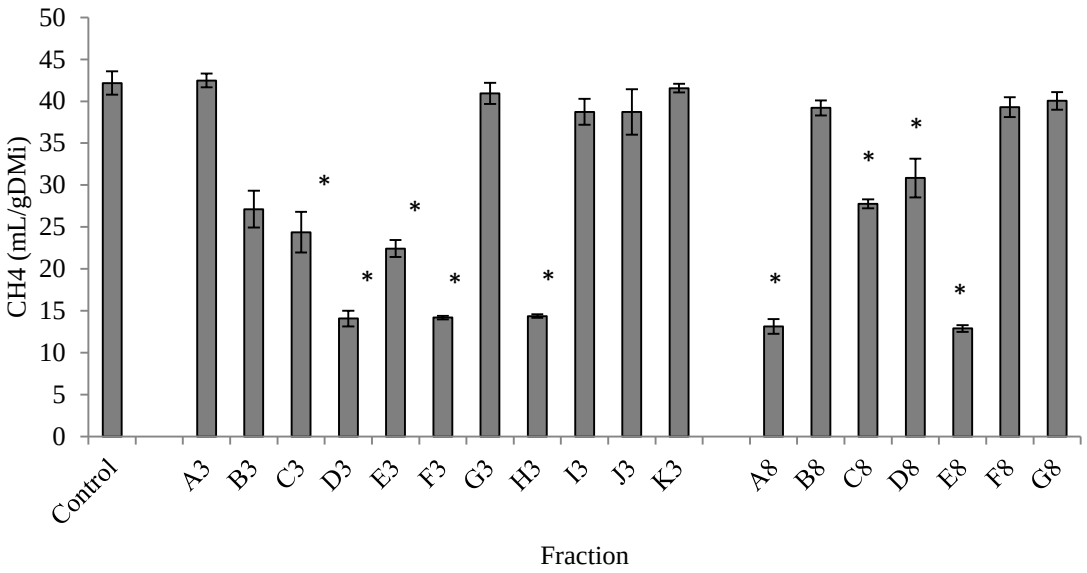


Figure 2.2. Methane production (mean ± SEM) by rumen microbes when fermenting oaten chaff in presence of selected fractions *in vitro.* * significantly (P<0.05) lower than the control. DMi - dry matter incubated. Control - oaten chaff + EtOH

Fermentation profiles of treatments are listed in Table 2.2. Briefly, gas and VFA were affected in 10 fractions. The fractions with the lowest methane, also had the highest reduction in these parameters, indicative of overall antibacterial effect.

Table 2.2. Fermentation parameters by rumen microbes when fermenting oaten chaff in the presence of selected fractions *in vitro.* * significantly (P<0.05) lower than the control. DMi - dry matter incubated. Control - oaten chaff + EtOH

Fraction	Gas (kPa)		VFA (mmol/L)		NH_3 (mg/L)
Control	86		117		79
Fractions from 2b and 3					
A3	89		130		68
B3	44	*	75	*	138
C3	45	*	80	*	150
D3	31	*	61	*	127
E3	46	*	80	*	108
F3	31	*	59	*	123
G3	85		126		67
H3	31	*	61	*	124
I3	86		127		68
J3	90		122		77
K3	88		124		74
Fractions from 8					
A8	31	*	60	*	125
B8	85		120		76
C8	47	*	85	*	160
D8	52	*	89	*	163
E8	29	*	66	*	126
F8	86		140		68
G8	85		122		74

The most potent fractions - D3, F3, H3, A8 and E8 were subjected further to HPLC-MS profiling that revealed a mix of compounds (Figures 2.3 and 2.4).

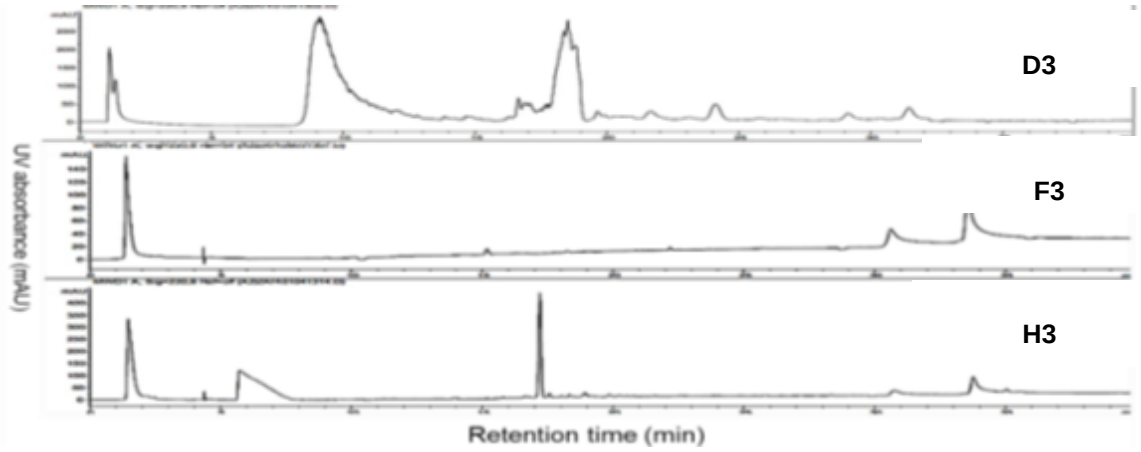


Figure 2.3: HPLC-MS of the fractions D3, F3 and H3

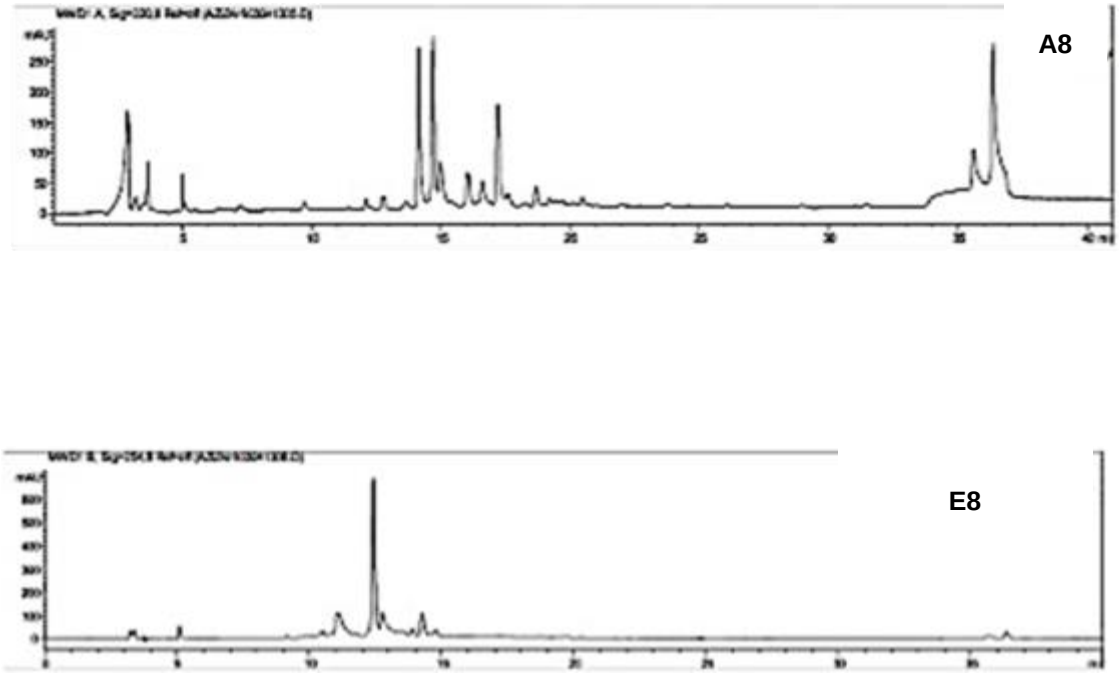


Figure 2.4: HPLC-MS of A8 and E8

Fractions D3, F3, H3 and A8, E8 were selected for further testing on basis of their strong antimethanogenic potential.

Experiment BA 19 - Third fractionation - fractionation of D3

Aim: to fractionate and test IVFT fraction D3

Results and discussion

Fraction D3 was re-fractionated to 3 fractions: D-13, D-14 and D-15. Methane production in all three was significantly reduced when compared to the control, but there were no significant differences in antimethanogenic activity among the fractions (Figure 2.5).

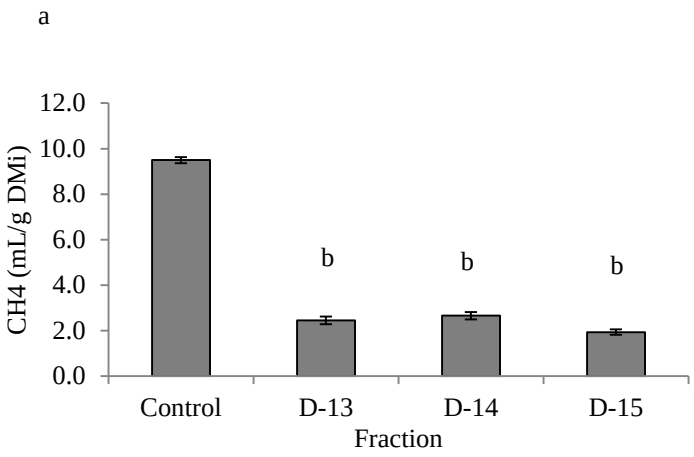


Figure 2.5: Methane production (mean ± SEM) by rumen microbes when fermenting oaten chaff in presence of selected fractions *in vitro.* * significantly (P<0.05) lower than the control. DMi - dry matter incubated. Control - oaten chaff + EtOH

In this experiment, only gas production was measured as indicator of overall antimicrobial effect, and none of the threefractions inhbited gas (Table 2.3). This finding is interesting, as the original fraction D3 in fact did inhibit gas and VFA. This demonstrates that it is possible to 'dilute out' negative effects of compounds on rumen microbial fermentation.

Table 2.3. Gas production by rumen microbes when fermenting oaten chaff in presence of selected fractions *in vitro.* * significantly (P<0.05) lower than the control. DMi - dry matter incubated. Control - oaten chaff + EtOH

Treatment	Gas (kPa)
Control	70
D-13	72
D-14	71
D-15	72

Experiment BB06 - Confirmation of the activities of crude extracts and fractions

Aim: to confirm activities of fractions obtained from new material prior to pure culture work

Background

In parallel to fractionation experiments, we conducted testing of selected fractions in pure culture. While initially this step was going to be done as a sequence to fractionation work and the most active and most advanced fractions were going to be used in pure culture testing, due to some delays in obtaining fractions in time and in enough quantities for pure culture testing, (as explained earlier), we have opted to test the most potent fractions from the first fractionation.

For this purpose, additional plant material needed to be sourced and therefore it was necessary to re-extract and confirm in the IVFT if the activities of extracts and fractions align to what was observed before.

Results and Discussion

Antimethanogenic activity was observed with plant material, ethanol, methanol, methanol/chloroform extracts, as well as with fractions 2, 8 and 9 (Figure 2.6). This aligned with our previous findings and the fractions 2, 8 and 9 were selected for testing in pure culture. Fraction 3was not active, but it was also included in further testing as an inactive fraction or 'negative' control.

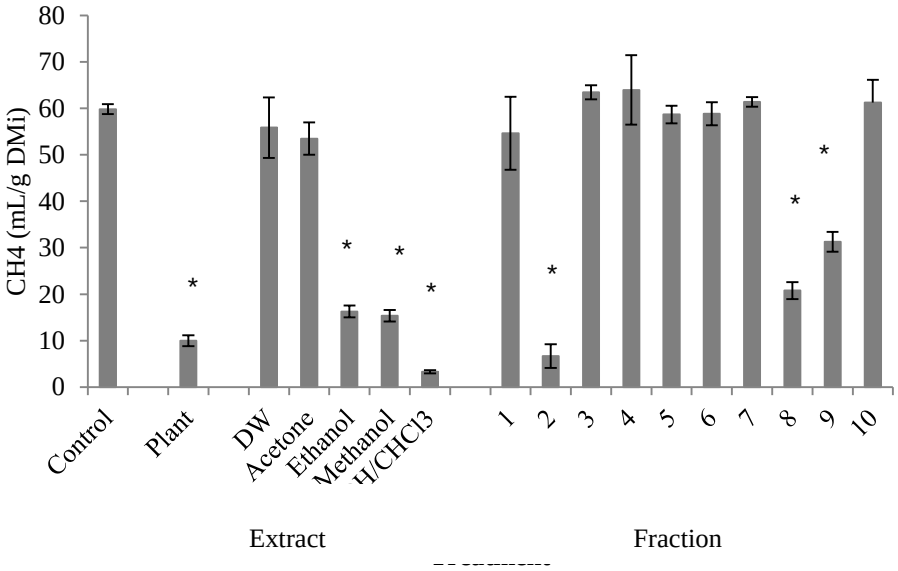


Figure 2.6. Methane production (mean ± SEM) by rumen microbes when fermenting biserula plant or oaten chaff in presence of selected extracts or fractions *in vitro.* * significantly (P<0.05) lower than the control. DMi - dry matter incubated. Control - oaten chaff + EtOH

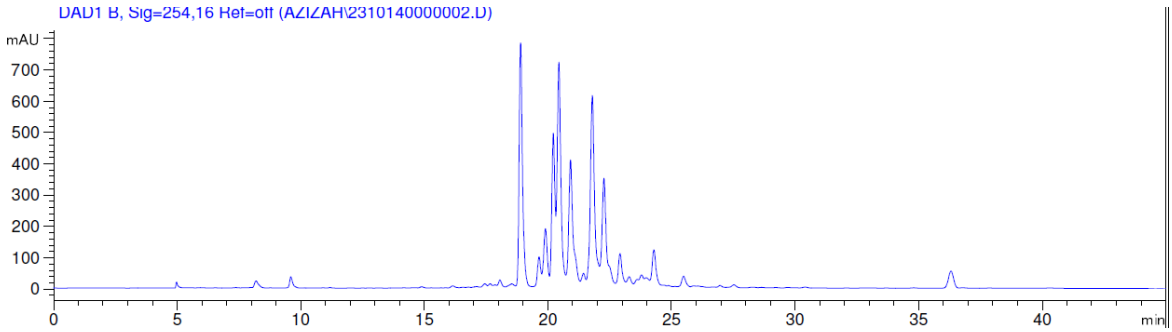
<u>Experiment BA/ELLE - HPLC profiling of fermentation liquid from RUSITEC fermentation of *B. pelecinus*</u>

Aim: to examine the HPLC profiles of crude extract of plant *B. pelecinus* and compare it to HPLC profiles of fermentation liquid when plant is digested by rumen microbes in the RUSITEC.

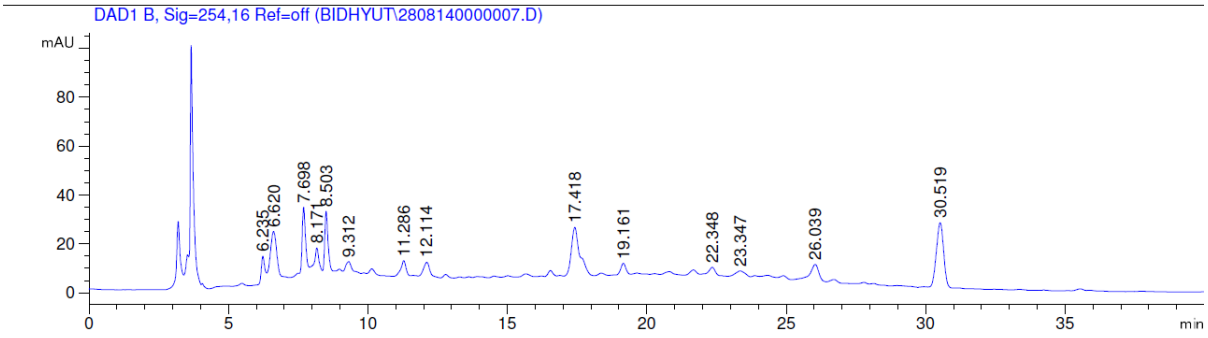
Results and discussion

There were some significant differences in HPLC profiles between the plant extract and the fermentation fluid, and the peaks became more diverse after exposure to rumen microbes (Figure 2.7). There were also some differences between sampling days. For example, a peak appearing at around 17.5 min was present at day 10, but not at day 20.

a



b



c

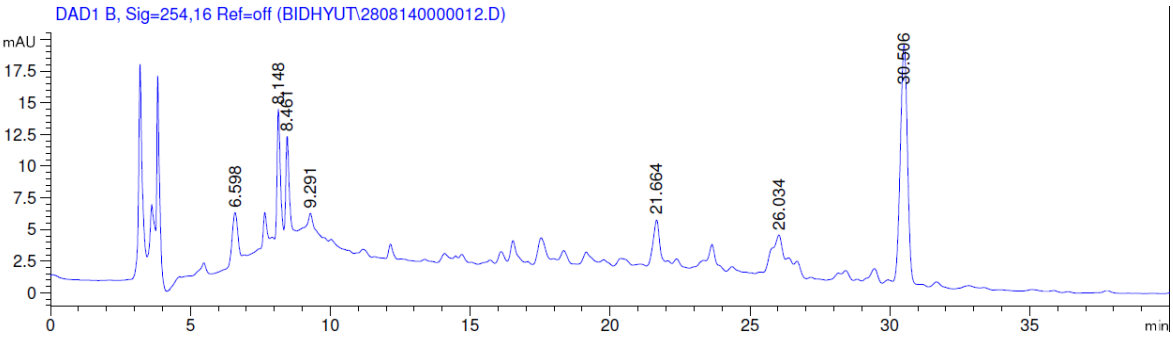


Figure 2.7. HPLC profiles of biserrula plant extract (a) and fermentation liquid on day 10 (b) and day 20 (c)

These findings confirmed that plant secondary chemistry changes when it is exposed to rumen microbes. It has also demonstrated that metabolite profiles may change over time when exposed to rumen microbes. This may potentially be a first sign of microbial adaptation and of a dormant potential of microbes to degrade these compounds that was triggered when exposed to the compound. This may explain why the treatment where the samples were taken became less effective over time, but further detailed analyses are needed to confirm this.

Discussion

With the current studies, we have demonstrated that extractable compounds in *B. pelecinus* are responsible for its antimethanogenic effect. Highly potent fractions were isolated and their effect was confirmed in the batch assay as well as in a pure culture (described later in the document). The active fractions maintained the trend observed with biserrula plant, i.e. reduction in methane was accompanied with reduction in overall gas production, but without affecting VFA or NH_3 production. However, with the last round of fractionation, i.e. with fraction D3 we have observed a potent antimethanogenic effect without reduction in gas. It is therefore possible to 'dilute out' some negative effects of bioactive plants.

Unlike *E. glabra*, within this project we were not able to isolate and identify any pure compounds in any of the fractions. It appears that the plant secondary profile in biserrula is very complex and that further studies are needed to pursue specific pure compounds in this plant responsible for the antimethanogenic effect.

Finally, we have confirmed that in biserrula, the plant chemical profile changes after it has been exposed to rumen microbial fermentation. While it still remains unclear which specific compounds were responsible for the antimethanogenic effect, the HPLC profiles coupled with further fractionation and testing in IVFT may help guide further fractionations in this plant. Moreover, we have found that certain plant metabolites may dissipate over time when exposed to fermentation by rumen microbes. It is possible that this is the evidence of a latent potencyin microbes to adapt to particular plant compounds that are present in the system. While the distribution of secondary metabolites examined here may have value in narrowing the search for active metabolites, their occurrence and fate may also reflect microbial adaptations and may help in understanding particular microbial strategies embedded in the rumen system and predict potential adaptation *in vivo*.

Implications

The results from these experiments confirmed that extractable compounds from *B. pelecinus* can reduce methane production, but the pure compounds responsible for this effect have not yet been isolated. The fractions maintained similar effects to those observed with the plant itself (i.e. reduction in methane coupled with normal VFA production) and provide further evidence that this plant may be a viable option for developing a sustainable CFI methodology that does not impede animal production, either by using the whole plant or its extracts/fractions or compounds.

References

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Durmic, Z., N.W. Tomkins, C.A. Ramírez-Restrepo, X. Li, Vercoe, P. E., 2012. *In vitro* fermentative traits of selected forages in North Queensland, AAAP, Bangkok, Thailand.

4. *In vitro* testing of essential oils and essential oil compounds

Background

In our previous studies, we have observed that eight commercial essential oils from Australian plants caused significant reductions in methane production by microbes fermenting a substrate (Durmic et al., 2013). *Melaleuca ericifolia* and *M. teretifolia* were the most potent and inhibited methane production substantially (i.e. 75%). However, all of the EO also significantly reduced gas and VFA production when included at a single level, i.e. 2500 µL/L of incubation medium. It remained unclear if the effect is dose-related and if it can be diluted further and not inhibit fermentation, by inclusion of lower dose of EO. It is also unknown if the effects persist over time and what the mechanism of their action is, or which specific compounds are responsible for the antimethanogenic effect.

The objectives of the studies in this section were i) to examine EO further at selected doses; ii) to examine the effects of major PSC in the most effective EO; iii) to examine persistence and mechanism of the effect in the RUSITEC

Experiment BA 1 - Testing the dose of selected essential oils

Aim: to test selected essential oils at different doses in the IVFT

Materials and methods

Eight essential oils, described previously (Durmic et al., 2013) were selected for examination of the first objective. Inclusion levels selected were 625, 1250, 2500 and 5000 µL/L. Following the screening, one EO (*S. spicatum*) was selected and tested further at lower levels, i.e. 62.5, 125, 250 and 500 µL/L.

Results

There was a clear dose response in all fermentation parameters (R^2 = 0.6-0.9). All EO inhibited methane at the highest dose (5000 µL/L), but only *L. pettersoni* and *S. spicatum* were effective at reducing methane at the lowest dose, i.e. 625 µL/L (Table 3.1). All reductions in methane were associated with significant reduction in gas, VFA and NH_3 concentrations; except for *S. spicatum* where gas was maintained at all levels of inclusion.

Table 3.1. Fermentation parameters from *in vitro* incubation of oaten chaff with 8 essential oils at selected doses. Control - concentrate diet (Milne EasyOne, Milne Feeds, WA). DMi - dry matter incubated. * significantly lower than the control (P < 0.05)

Parameter	Dose (µL/L)					
	Control	625	1250	2500	5000	SEM
A.fragrans						
Gas (mL/g DMi)	280	250	248	221 *	161 *	4.2
CH_4 (mL/g DMi)	25	27	25	21	5 *	0.5
VFA (mmol/L)	110	93 *	81 *	68 *	50 *	2.5
N-NH_3 (mg/L)	173	105 *	114 *	105 *	70 *	2.4
E.plenissima						
Gas (mL/g DMi)	280	298	281	232 *	152 *	3.6

Parameter	Dose (µL/L)					
	Control	625	1250	2500	5000	SEM
CH_4 (mL/g DMi)	25	37	36	22	7 *	0.6
VFAs (mmol/L)	110	98 *	91 *	68 *	47 *	1.6
N-NH_3 (mg/L)	173	136 *	102 *	109 *	60 *	2.2
L.pettersoni						
Gas (mL/g DMi)	280	232 *	195 *	193 *	102 *	15.4
CH_4 (mL/g DMi)	25	19 *	6 *	6 *	3 *	0.7
VFA (mmol/L)	110	62 *	50 *	45 *	41 *	2.0
N-NH_3 (mg/L)	173	88 *	61 *	42 *	50 *	2.7
M.alternifolia						
Gas (mL/g DMi)	280	270	219 *	160 *	138 *	6.9
CH_4 (mL/g DMi)	25	30	17 *	8 *	7 *	1.8
VFA (mmol/L)	110	85 *	78 *	54 *	47 *	2.0
N-NH_3 (mg/L)	173	129 *	134 *	106 *	81 *	3.2
M.ericifolia						
Gas (mL/g DMi)	280	245	197 *	178 *	41 *	4.1
CH_4 (mL/g DMi)	25	26	17 *	12 *	2 *	0.4
VFA (mmol/L)	110	81 *	82 *	59 *	43 *	2.8
N-NH_3 (mg/L)	173	162	110 *	74 *	70.3 *	0.1
M.teretifolia						
Gas (mL/g DM)	280	252	195 *	149 *	29 *	2.6
CH_4 (mL/g DM)	25	28	6 *	5 *	1 *	0.2
VFA (mmol/L)	110	76 *	54 *	49 *	44 *	1.1
N-NH_3 (mg/L)	173	74 *	50 *	37 *	56 *	1.5
S.spicatum						
Gas (mL/g DM)	280	261	254	248	249	4.5
CH_4 (mL/g DM)	25	17 *	14 *	13 *	13 *	0.6

Parameter	Dose (µL/L)					
	Control	625	1250	2500	5000	SEM
VFA (mmol/L)	110	87 *	87 *	93 *	89 *	1.5
N-NH_3 (mg/L)	173	118 *	95 *	102 *	71 *	3.0
L.angustifolia						
Gas (mL/g DM)	280	266	226 *	90 *	29 *	5.9
CH_4 (mL/g DM)	25	30	22 *	3 *	1 *	0.2
VFA (mmol/L)	110	91 *	72 *	48 *	44 *	1.8
N-NH_3 (mg/L)	173	122 *	105 *	68 *	71 *	2.8

When *S. spicatum* was tested at lower doses, a significant reduction (12% compared to control) in methane was maintained even at the lowest dose (62.5 µl/L, Table 3.2). In this experiment, there was also no reduction in gas at all inclusion levels, while VFA and NH_3 were only inhibited at the highest dose (500 µl/L).

Table 3.2. Fermentation parameters from *in vitro* incubation of oaten chaff with *S. spicatum* at selected doses. Control - Milne EasyOne, Milne Feeds, WA. * significantly lower than the control ($P < 0.05$)

Fermentation parameter	Dose (µl/L)				
	Control	62.5	125	250	500
Gas (mL/g DMi)	362	364	360	359	350
Methane (mL/g DMi)	33	29 *	28 *	28 *	24 *
Total VFA (mmol/ L)	97	94	91	92	90 *
N-NH_3 (mg/L)	319	291	272	282	226 *

Discussion

The results from the current study show that selected EO had potent antimethanogenic effects, but also general antimicrobial effect, as judged by the reduction in microbial fermentation parameters. The most effective EO were*L. pettersoni*, *M. alternifolia* and *M. ericifolia*, which supports our previous findings (Durmic et al., 2013). *S. spicatum* was also highly effective, causing up to 50% reduction (with the highest dose) while not affecting gas production (at all doses), and other fermentation parameters when included at dose of 250 µL/L or lower. The effect in all EO was dose-related, but it was demonstrated that it was possible to dilute the antimicrobial effect with lower doses in *S. spicatum*.

Experiment BA 2 - Testing pure compounds from selected essential oils

Aim: to examine selected essential oil compounds in the IVFT

Materials and methods

M. teretifolia, *M. ericifolia* and *L. pettersoni* were selected for further testing, based on their effectiveness and commercial availability of essential oil compounds. Five commercially available compounds found as main compounds in these EO (Table 3.3) were obtained from Sigma Aldrich and tested at 62.5, 125, 250 and 500 µL/L.

Table 3.3. List and source of major secondary compounds of *Melaleuca teretifolia*, *Melaleuca ericifolia* and *Leptospermum pettersoni* [http://www.paperbarkoils.com.au/essential-oils.html#h1; (Southwell et al., 2003; Kim et al., 2012)]

Essential oil	Compound tested	Amount in EO	Source
Melaleuca ericifolia	'L'	50%	L2602 Aldrich
Melaleuca teretifolia	Eucalyptol	84%	W246506 Aldrich
	Terpinen-4-ol	1.8%	W304506 Aldrich
	'C'	4.0%	W230308 Aldrich
Leptospermum pettersoni	(±) Citronellal	16%	W230715 Aldrich

Results

In this experiment, all compounds tested, except for eucalyptol, caused a significant reduction in methane (Table 3.4). The most potent one was L at the lowest inclusion level (6.25 µL/L), causing about 30% reduction in methane when compared to the control. Only some treatments and at highest inclusion level (500 µL/L) caused reduction in VFA and NH_3 concentrations, while none affected gas production.

Table 3.4. Fermentation parameters from *in vitro* incubation of oaten chaff with selected pure compounds from EO. DMi - dry matter incubated. Control - concentrate diet. * significantly lower than the control ($P < 0.05$)

Parameter	Dose (µL/g DMi)				
	Control	6.25	12.5	25	50
L					
Gas (mL/g DMi)	362	355	358	357	346
CH_4 (mL/g DMi)	24	17 *	20 *	20 *	19 *
VFA (mmol/L)	97	96	99	100	95
N-NH_3 (mg/ L)	319	271	277	269	201 *
Eucalyptol					
Gas (mL/g DMi)	362	366	366	366	367
CH_4(mL/g DMi)	24	24	24	23	25

Parameter	Dose (µL/g DMi)				
	Control	6.25	12.5	25	50
VFA (mmol/L)	97	121	98	103	111
N-NH_3 (mg/ L)	319	348	348	341	330
Terpinen-4-ol					
Gas (mL/g DMi)	362	355	355	356	350
CH_4 (mL/g DMi)	24	20 *	20 *	20 *	20
VFA (mmol/L)	97	104	104	110	99.6
N-NH_3 (mg/ L)	319	291	287	296	296
'C'					
Gas (mL/g DMi)	362	362	365	362	351
CH_4 (mL/g DMi)	24	21 *	21 *	21 *	21 *
VFA (mmol/L)	97	91	97	100	87 *
N-NH_3 (mg/ L)	319	287	296	293	191 *
(±) Citronellal					
Gas (mL/g DMi)	362	362	351	353	341
CH_4 (mL/g DMi)	24	21 *	20 *	21 *	17 *
VFA (mmol/L)	97	100	102	98	98
N-NH_3 (mg/ L)	319	272	220 *	279	227 *

Discussion

The results from the current study implied that several purified compounds from Australian EO are capable of inhibiting methane without inhibiting overall fermentation. One of these, i.e. L, a major compound in *M. ericifolia* was found to be the most effective. This candidate will be tested further to confirm the effect and reveal persistency, adaptation and mechanism in the Rusitec, as well as in the pure culture of methanogens. Theresults from this study also confirmed that it is possible to achieve significant reduction in methane without affecting rumen microbial fermentation.

Implications

Selected EO as well as some purified compounds (i.e. L) from Australian plants may be potent methane inhibitors. It is possible to achieve significant reduction in methane with these without affecting rumen microbial fermentation by carefully selecting doses.

References

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Experiment BA 3 - Assessment the effects of *Melaleuca ericifolia* essential oil and its main secondary compound L on ruminal fermentation of a concentrate-based diet in the RUSITEC

Background

In our previous experiments (BA1 and BA2), we identified a suite of essential oils, and their major secondary compounds, that can inhibit methane production from microbes during *in vitro* fermentation of a concentrate-based diet. In that respect, *Melaleuca ericifolia* and its major secondary compound L, were found to be the most effective. L, at the lowest inclusion level tested (i.e. 62.5 µL/L), caused about a 30% reduction in methane, without affecting gas or VFA and NH_3 production when compared to the control.

The aim of the present study was to investigate the effect of supplementation of *M. ericifolia* and/or L on the *in vitro* rumen fermentation characteristics, fermented plant chemical profiles and microbial population profile of a high concentrate diet in RUSITEC.

Materials and methods

The experiment was conducted using rumen simulation technique (RUSITEC) as described by Czerkawski and Breckenridge (1977). Nine 650-ml effective volume vessels were used in this experiment. Each fermentation vessel was filled with 500 mL of warmed McDougall's buffer [(pH 8.2; McDougall, 1948) modified to contain 1.0 g/L of $(NH_4)2SO_4$] and 150 mL of filtered rumen fluid. In addition, a bag (7 x 17 cm, 52 µm pore size) contained solid rumen digesta (20 g) and a bag contained experimental diet (15 g, dry matter basis) were placed within each fermentation vessel. A concentrate-based diet (EasyOne, Milne Feeds, Welshpool, Australia; 14.5% CP; ME 11.0 MJ/kg; crude fibre 20.0%), ground to pass through 2-mm screen, was used as a substrate. Rumen fluid was obtained from pooled ruminal contents collected from three ruminally fistulated adult Merino ewes, at 2 h after the morning feeding. Animals were fed with 0.6 kg of oaten chaff and 0.4 kg of lupins (per

head/day). Ruminal content was immediately transferred to the laboratory in a pre-warmed thermos then strained through four layers of cheesecloth to eliminate large feed particles. Animals were managed according to protocols approved by the University of Western Australia Animal Care and Use Committee. After 48 h, the solid rumen digesta bag was replaced with a polyester bag containing either 15 g of experimental diet. Thereafter, 2 nylon bags were replaced daily; allowing each bag to remain in the fermenter for 24 h. Each vessel was 'fed' daily at 0900 h with new bags containing the experimental diet. Artificial saliva was continuously infused into the fermenters at a dilution rate of 4%/h. During nylon bag exchange, each fermentation vessel was flushed with approximately 2 L of CO_2 to maintain anaerobic conditions.

Three experimental treatments - *M. ericifolia* essential oil (MEE), L and a control without additives were allocated to the experimental units (vessels) according to a completely random design, with 3 replicates per experimental treatment. The experimental period consisted of 19 d, whereby the first 7 d (d 1 to 7) served as an adaptation period to allow for equilibration of microbial populations, followed by 12 d of sampling and measurement (day 8 to 19). In the adaptation period, all fermenters were supplied with control diet only. From day 8 onwards, *M. ericifolia* essential oil and L were added daily, directly to the fermenter, at a concentration of 500 µL/L of culture ruminal fluid. The daily treatment dose was first dissolved in ethanol at 1:1 ratio, and the appropriate amount was added to the fermenter. The control treatment was dosed with an equivalent amount of ethanol.

The measurement period was divided into 3 stages [initial (day 8 to 11), mid (day 12 to 15), and final (day 17 to 19)]. The pH of the fluid from each fermenter was measured daily, immediately before feeding. Samples were also collected daily, 4 h post-feeding, to determine VFA and NH_3 concentrations in the fermentation liquid. Fermentation was arrested by adding 0.4 ml of 1M NaOH (for VFA analysis) or 0.4 ml of 2M HCl (for NH_3 analysis) to a 4 mL sample, taken directly from a port on the top of the fermentation vessels and immediately frozen at −20°C until analysis. Aliquots were also collected, filtered and subjected to GC-MS analysis.

Fermentation gas was collected into reusable 4000 mL silver wine bladders attached to each fermenter. Just prior to feed-bag exchange, daily (24 h) total gas production from each fermenter was determined using a gas flow meter. In each collection period, just prior to determination of total gas, methane concentrations were measured directly from the bag using a GC (Varian MicroGC).

Feed bags that were removed from fermenter vessels, were washed in cold tap water until the water running off was clear and then the bags were dried at 60°C for 48 h. The apparent dry matter (DM) disappearance at 24 h was calculated from the loss in weight, and the residues were analyzed for crude protein (CP), neutral detergent fibre (NDF) and acid detergent fibre (ADF) to estimate CP, NDF and ADF apparent disappearance.

The predominant microbial members associated with fibre digestion, hydrogen, carbon dioxide and methane production in the rumen, i.e. *Fibrobacter succinogenes, Ruminococcus albus* & *Ruminococccus flavifaciens* and methanogens *Methanobrevibacter* spp. (*M. ruminantium, M. gottschalkii, M. thaueri* and *M. olleyae* etc) and those belonging to the new VII order '*Methanoplasmatales*' (member of the so-called rumen cluster 'C', or RCC) were also assessed. In this study, we used quantitative PCR to interrogate the relative contribution of both fibrolytic bacteria and methanogens in the samples.

Data were analysed as repeated measures using the MIXED procedure of SAS (9.1). The MIXED model accounted for the repeated measures (sampling day), the fixed effects of treatment, and the interaction between treatment and sampling day. Results are reported as least squares means.

Results

Overall, addition of *M. ericifolia* essential oil or L resulted in a decrease (P<0.05) in total gas, methane production, apparent DM disappearance and NH_3, and an increase in pH when compared with the control, with L having a more potent effect compared to MEE treatment. L also reduced total VFA significantly, in particular propionate, while concentrations of NH_3 were reduced with both MEE and L (Table 3.5).

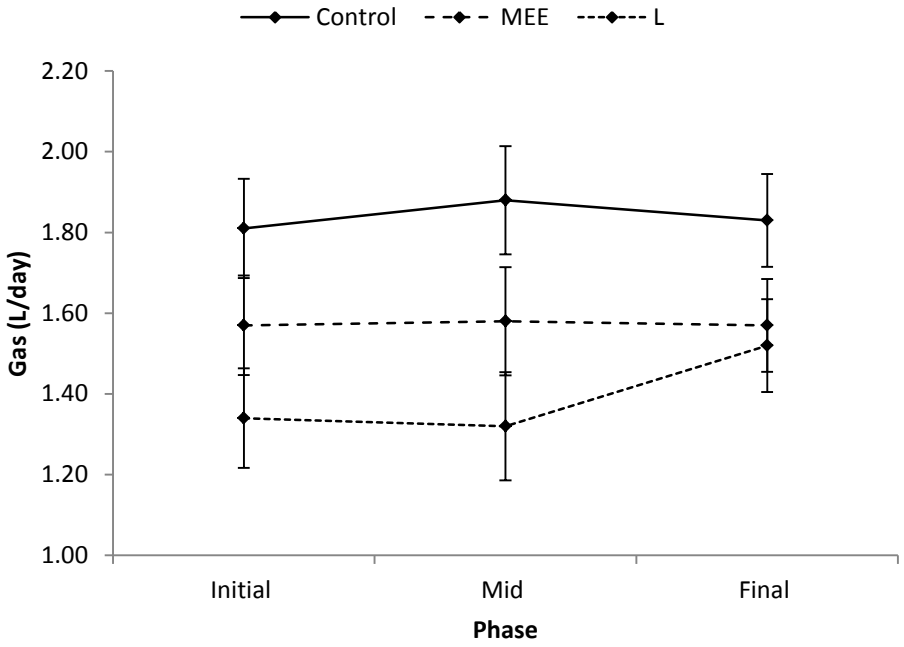
Table 3.5. Effect of *Melaleuca ericifolia* essential oil (MEE) and L on ruminal fermentation characteristics of a high concentrate diet measured using a RUSITEC system (mean ± SEM for whole experimental period). [1]-Probabilities associated with *F*-tests for treatment (Treat), sampling period, and the interaction between treatment and sampling period. Within each row, values not sharing the same superscript vary (P<0.05). BCFA - branched chain fatty acids

Fermentation parameter	Treatment				Effect[1] (*P*)		
	Control	MEE	L	SEM	Treatment	Period	Treatment x Period
Gas production (ml/ 24 h)	1.84[a]	1.57[b]	1.39[c]	0.071	<0.01	0.60	0.59
Methane (ml/24 h)	153[a]	137[b]	126[b]	5.50	<0.01	<0.01	<0.01
DM disappearance (g/g)	0.56[a]	0.54[b]	0.52[c]	0.005	<0.01	<0.01	<0.01
pH	6.91[a]	7.0[b]	7.1[c]	0.017	<0.01	<0.01	0.07
NH_3 (mg/ L)	439[a]	281[b]	343[c]	13.0	<0.01	<0.01	<0.01
Total VFA (mmol/L)	106[a]	107[a]	87.6[b]	6.45	<0.05	0.97	0.99
Acetate : propionate	3.74	4.18	4.86	0.73	0.34	<0.01	0.97
Individual VFA (mol/100 mol)							
Acetate	52.9	53.1	53.8	0.67	0.43	<0.01	0.74
Propionate	18.3[a]	16.5[a]	13.8[b]	1.01	0.03	<0.01	0.87
Butyrate	15.7	16.1	16.5	0.49	0.50	0.01	0.98
BCFA	2.20[a]	1.33[b]	1.47[b]	0.32	0.04	0.72	0.98

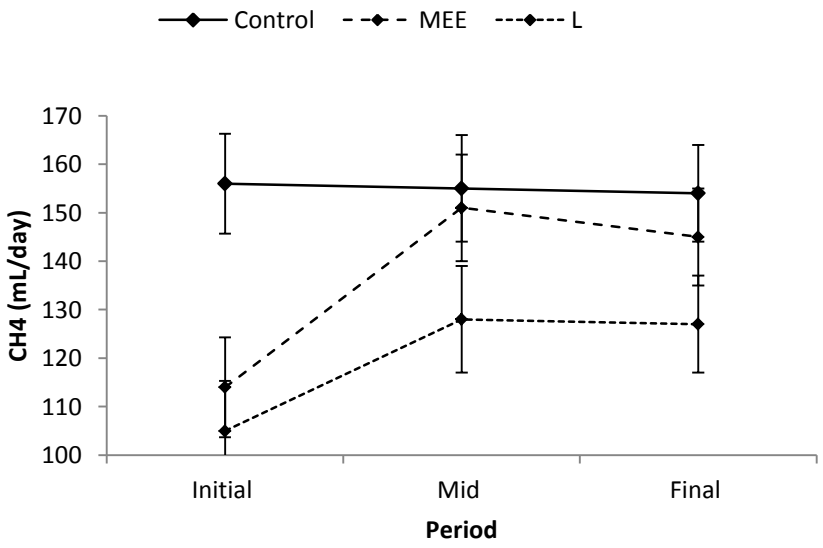
The effect of MEE and L on fermentation were persistent throughout three periods, but the differences with the control became smaller towards the end for gas (L), methane (both), apparent DM

disappearance (MEE) and VFA (both, Figure 3.1a,b). L resulted in a decrease in apparent DM disappearance in all three measurement stages (P<0.05; Figure 4.1c), but significantly suppressed VFA at the initial collection period (Figure 4.1d).

a



b



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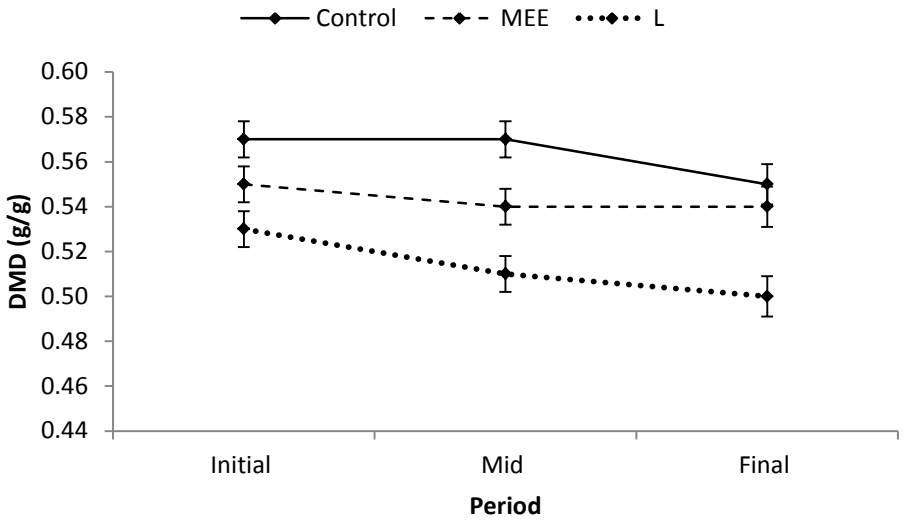

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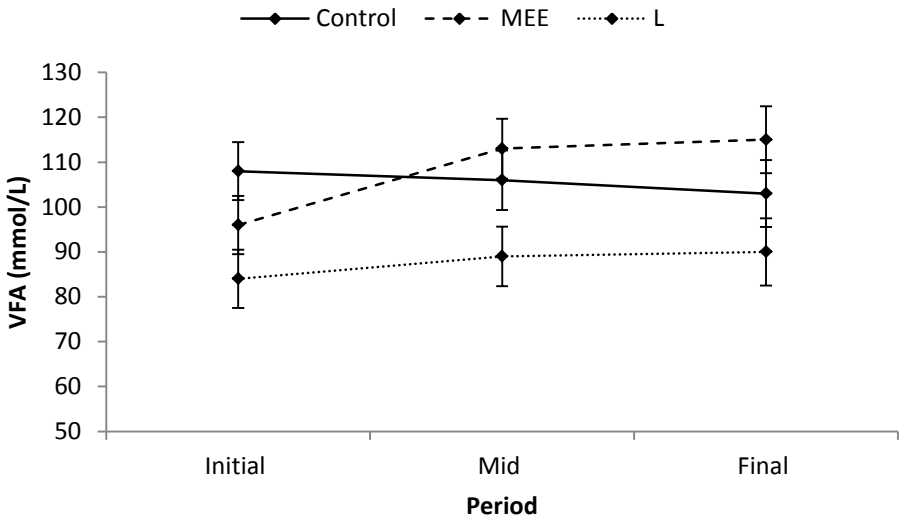


Figure 3.1. Average gas production (a), methane production (b), apparent DM disappearance (c) and VFA (d) across three different phases of measurement. SEM - overall SEAM for treatment for the whole period.

The GC-MS analysis confirmed that L was the major compound detected in *M. erecifolia*, as well as in fermentation liquids of *M. erecifolia* and L treatments (Table 3.6). Some other compounds found included octanoic acid, cyclohexane and hexanoic acid. L was present in both treatments throughout the samplings, but based on % area in GC-MS profiles of fermentation liquid, somewhat higher at the end compared to beginning in both treatments.

Table 3.6. Plant secondary compounds (% area on GC-MS) in pure additives (PA) - EO (MEE) and EOC (L) and in fermentation fluids (FF) from these treatments in the RUSITEC

Compound	MEE			L		
	PA	FF Beg	FF End	PA	FF Beg	FF End
L	59	41	67	100	31	42
Octanoic Acid		11				
Hexanoic acid		18			10	
lp0h a.-Methyl-.alpha.-[4-methyl-3-pentenyl]oxiranem					11	11
lp3h a.-Methyl-.alpha.-[4-methyl-3-pentenyl]oxiranem					11	11
prop[e]azulene, decahydro-1,1,7-trimethyl-4-m	20			-		
cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl	13			-		

The qPCR analysis revealed that the numbers of both the fibrolytic bacteria, *F. succinogenes* and *R. flavifaciens,* did not change significantly or were slightly increased for only MEE (Figure 3.2 an 3.3). *R. albus*, however, was inhibited by both treatments when averaged for all time points. However, when analysed for each compound separately, *R. albus* numbers improved on days 15 & 19 compared to day 11. Methanogen numbers decreased across days 11, 15 & 19, for RCC. Other methanogen targets, mcrA (total methanogens) & Mbr (*Methanobrevibacter*) were either lower or, not significantly higher than controls for the same periods (Figure 3.4).

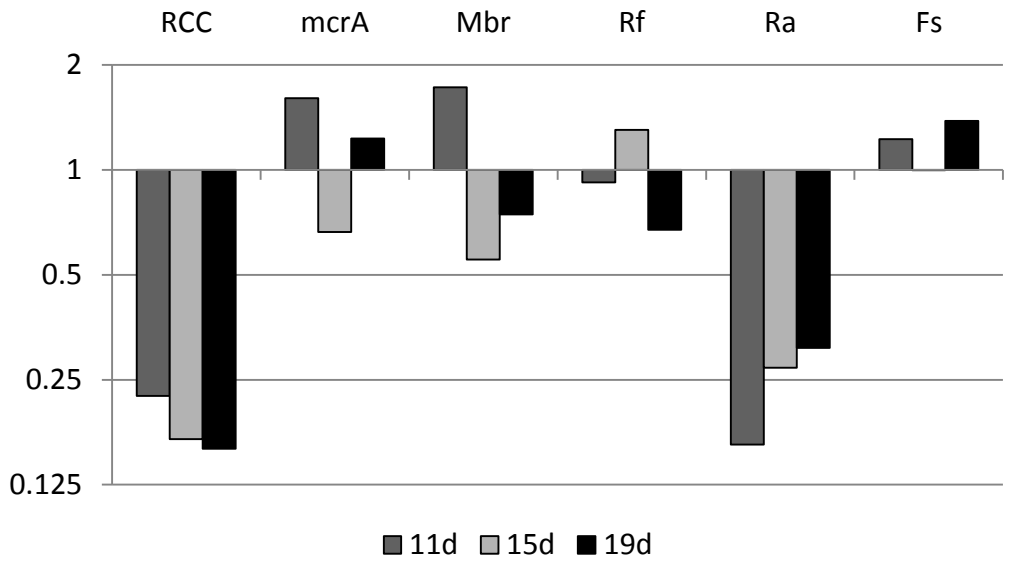


Figure 3.2. Total numbers of methanogens and bacteria in L treatments as determined by qPCR. Total bacterial numbers were used as reference for each time period. (Note: 0.5 = 2x; 0.25 = 4x; 0.125 = 8x and 0.1 = 10x reductions). RCC - Rumen Cluster C methanogens; mcrA - Methyl coenzyme-M reductases gene product; *Methanobrevibacterspp*; Rf - *Ruminococcusflavefavciens*; Ra - *Ruminococcusalbus*; Fs - *Fibrobactersuccinogenes*

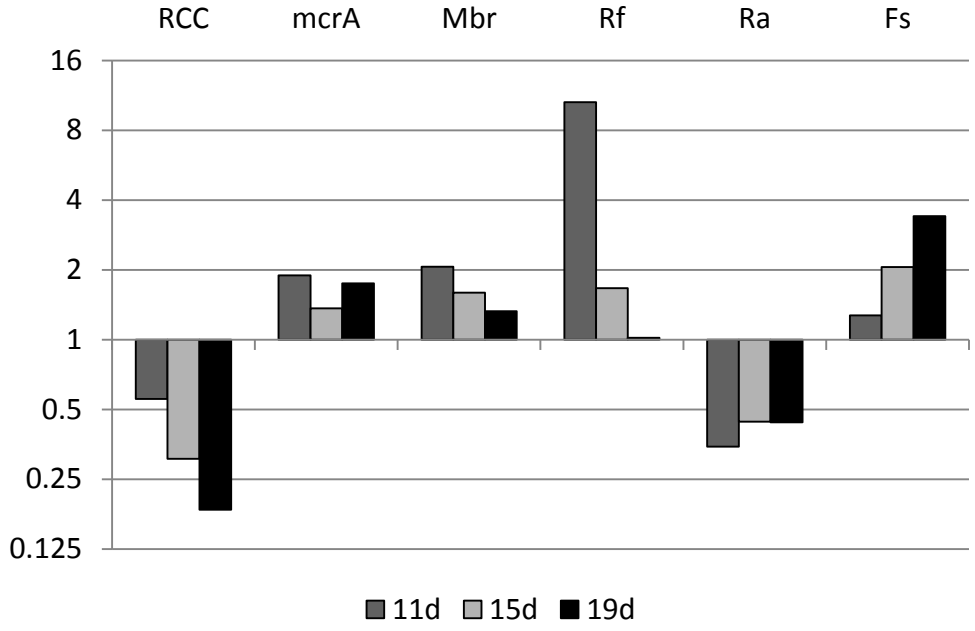


Figure 3.3. Total numbers of methanogens&bacteria in MEE treatments as determined by qPCR. Total bacterial numbers were used as reference for each time period. (Note: 0.5 = 2x; 0.25 = 4x; 0.125 = 8x and 0.1 = 10x reductions). RCC - Rumen Cluster C methanogens; mcrA - Methyl coenzyme-M reductases gene product; *Methanobrevibacterspp*; Rf - *Ruminococcusflavefavciens*; Ra - *Ruminococcusalbus*; Fs - *Fibrobactersuccinogenes*

Table 3.7. Mean fold reduction in gene expression ($2^{-\Delta\Delta CT}$ values, relative to control)

Treatment	RCC	*R.albus*	RCC (SE)	Ra (SE)
MEE	0.349	0.41	0.06	0.04
L	0.184	0.247	0.02	0.02

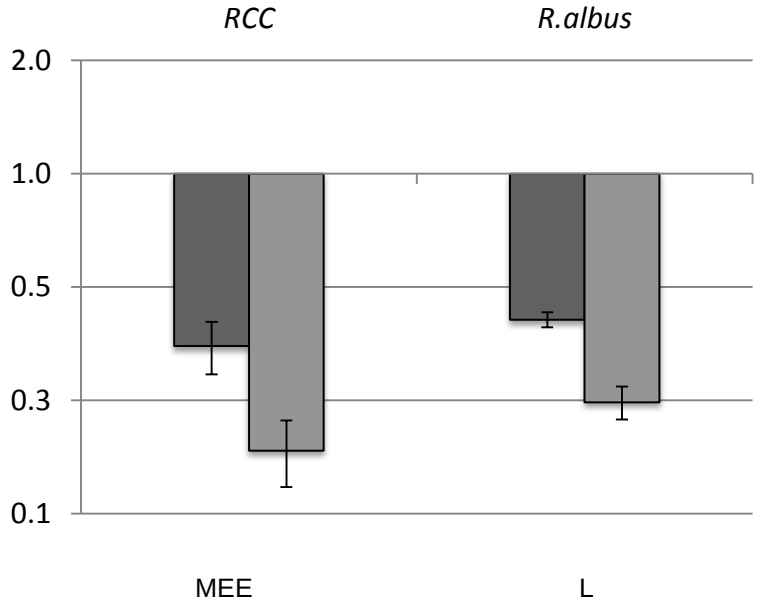


Figure 3.4. Mean Fold reduction of RCC &*R. albus* for the combined three periods relative to controls. Total bacteria was used as a reference for each time period. (Note: 0.5 = 2x; 0.25 = 4x; 0.125 = 8x and 0.1 = 10x reductions).

Next-Generation Sequencing (NGS) Analysis

Analysis of the results from unweighted Unifrac analysis by Principal Coordinate Analysis (PCoA) plots clearly separated samples based on the treatment (Figure 3.5). Maximum diversity in this instance, is along the PC1 axis (48% dissimilarity) and the PC2 (11% dissimilarity), with the L cluster showing the maximum divergence along both axes. The MEE samples, however, were spread mostly midway along the PC1 axis and lowest in the PC2 axis and form a less divergent cluster. The Control samples were tightly clustered with a small spread along the PC2 axis. These were also the least divergent for all 3 time periods. All samples also have a slight spread along the PC3 axis as well.

When the treatment and fermentation days are analysed together, DL treatment for D4 & D8 formed a loose cluster that are uniquely distant from D12, which showed maximum divergence along both PC1 and PC2 axes. Among the MEE samples, clustering was less clear-cut in that only 2 samples each from D8 & D12 that showed maximum divergence, clustered loosely whereas control samples with one each from D8/D12 formed a tight group with low dissimilarity (Figure3.6)

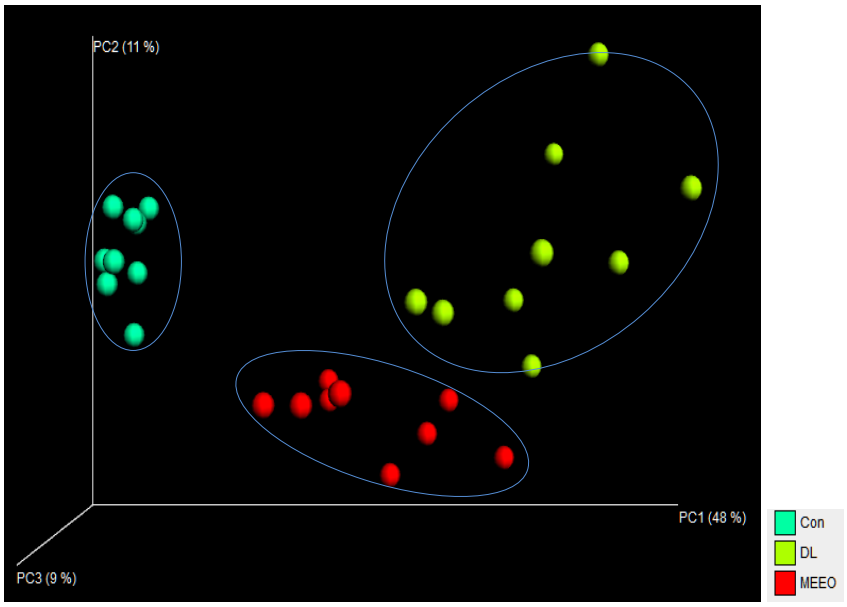


Figure 3.5. Principal Component Analysis (PCoA) plot using unweighted Unifrac analysis showing distribution of taxonomic diversity of microbial OTUs from Control, L and MEE treatments in the RUSITEC

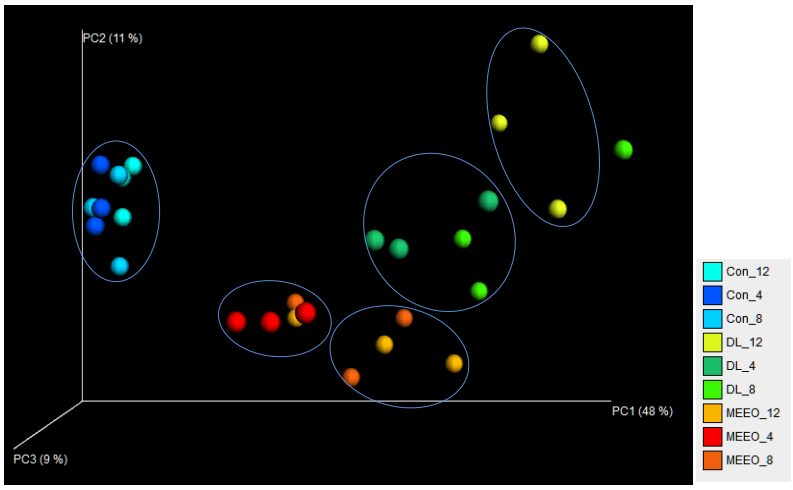


Figure 3.6. Principal Component Analysis (PCoA) plot using unweighted Unifrac analysis showing distribution of taxonomic diversity of microbial OTUs from Control, L & MEE treatments in the RUSITEC at different days (D4, D8 & D12)

Analysis of Operational Taxonomic Units (OTUs) based on the percentage of phylogenetically assigned sequence reads resolved at varying depths (phylum level to genus level) showed the diversity and richness of the microbial targets. At the phylum level, *Firmicutes* dominated generally in all three treatments, whereas there was a marked increase of *Actinobacteria* and a decrease in or none at all of *Bacteroidetes* sequences in both L & MEE samples. Similarly, *Synergistetes*, *Spirochetes* and *Proteobacteria* were missing in both the L & MEE treatments (Figure 3.7a). A separation of the three time points however, showed that *Acinobacteria* and *Bacteroidetes* progressively diminished with a concomitant increase in *Firmicutes* as time progresses. The

Synergistetes and the *Proteobacteria* increased progressively in the control only without being represented in the L and MEE treatments (Fig 3.7b). *Archaea* including methanogens were consistently represented in all three treatments, with an increase in the MEE treated samples.

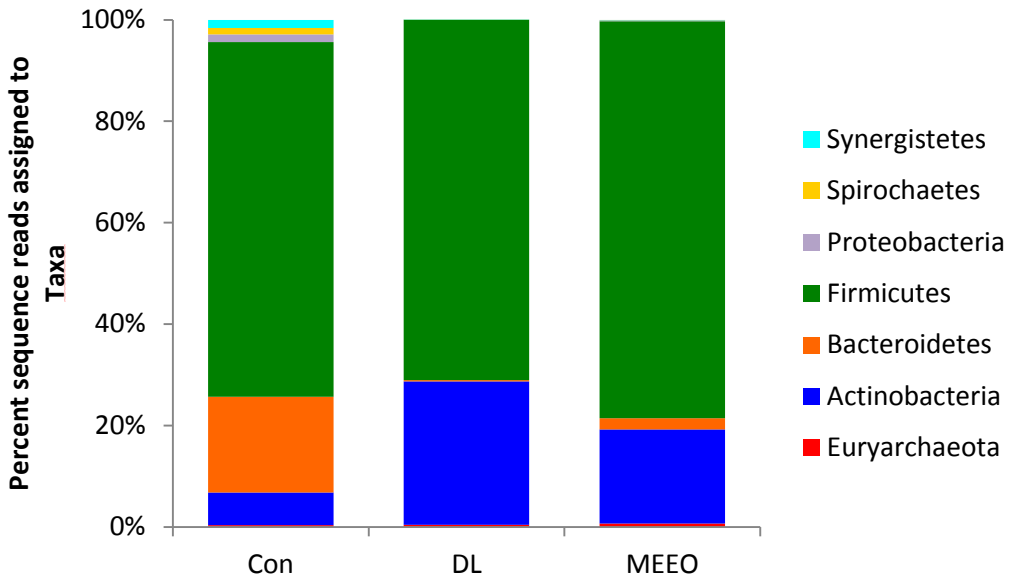


Figure. 3.7a. Representation(%) of taxonomically assigned microbial OTU reads (Illumina MiSeq) at the phylum level on Control, L & MEE treatments in the RUSITEC

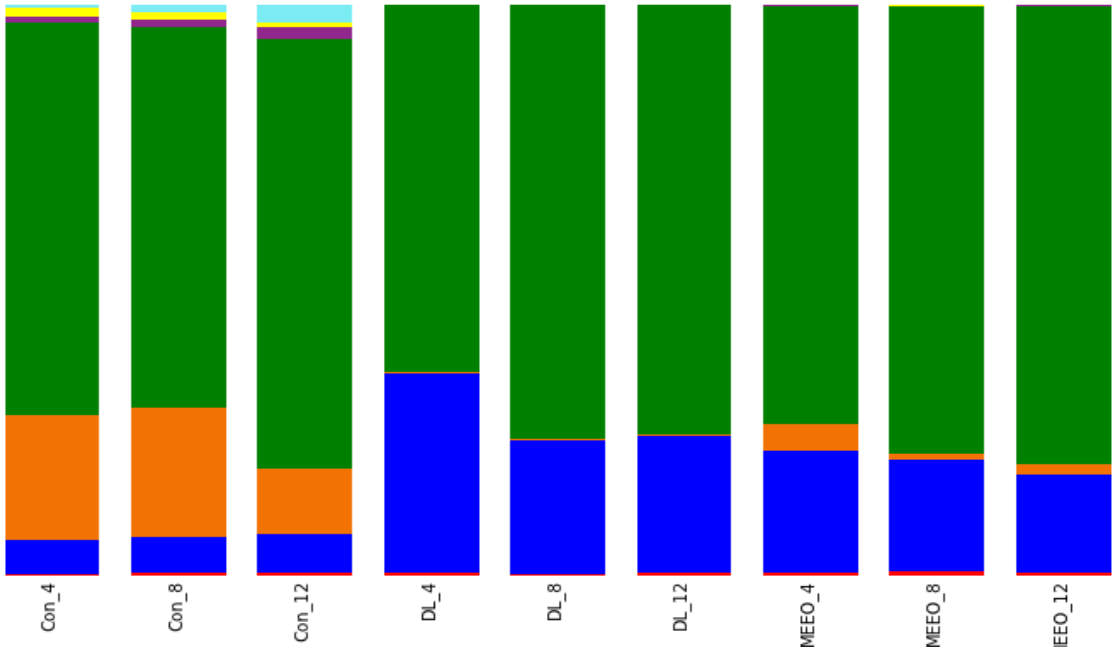
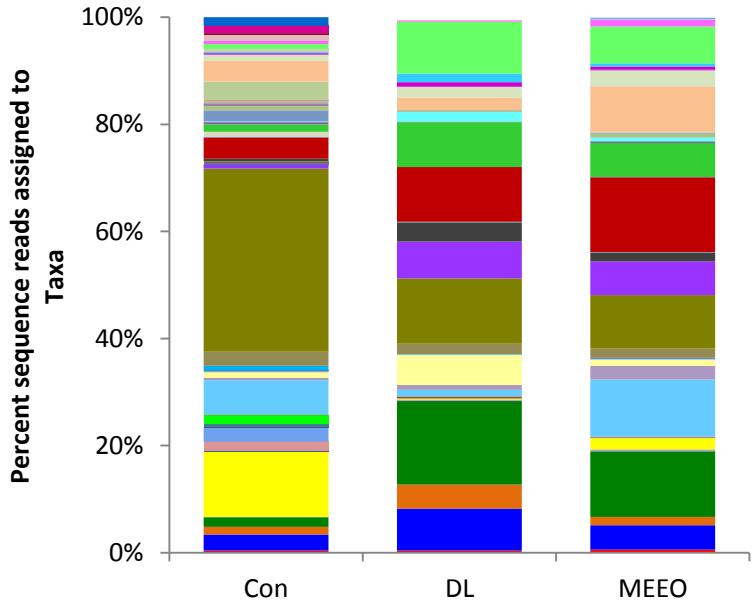


Figure. 3.7b. Representation (%) of taxonomically assigned microbial OTU reads (IlluminaMiSeq) showing time effect at the phylum level on Control, L & MEE treatments in the RUSITEC at different sampling times (D4, D8 & D12)

Analysis of sequence distribution at the genus level (97% species cut-off) showed the diversity of the OTUs between control and treatment samples (Figure 3.8a). Some of the OTUs could only be resolved at the family level, possibly due to presence of novel sequences not yet classed as genus. Bacterial OTUs that have significantly increased in both the treatments compared to the controls

include *Firmicutes* like *Bulleida* (*Erysipelotrichacea*), *Butyrivibrio,* unclassified *Clostridiales Lachnospiracea* and *Eubacterium*. Others like *Succiniclasticum* and increased only in MEE whereas *Streptococcus* declined rapidly in both the treatments. The phylum *Bacteroidetes* related OTUs tended to disappear completely in the L treated samples where as it was reduced drastically in the MEE treatments. Even in the controls this group decreased to ~25% of the starting numbers by the 19th day of fermentation (Figure 3.8b). Members of the *Coriobacteracea* (unclassified genus), however, increased from 1.8% to 15.6% & 12.2% in L and MEE treatments respectively. Similarly, *Bifidobacterium* and its unclassified family members increased, overall in L and MEE treatments, their numbers fluctuated during fermentation: with an increase in the *Bifidobacterium* genus while the unclassified *Bifidobacteriacea* declined or remained steady during the 3 periods. Others like *Prevotella* and *Paraprevotella* disappeared and *Firmicutes*like *Planococcacea* reduced with L treatment while increasing sharply under MEE treatment.



Taxonomy	Con	DL	MEEO
Methanobrevibacter	0.40%	0.40%	0.60%
Bifidobacteriaceae;g__	3.00%	7.80%	4.50%
Bifidobacterium	1.40%	4.50%	1.60%
Coriobacteriaceae;g__	1.80%	15.60%	12.20%
Bacteroidales;f__;g__	12.10%	0.20%	2.10%
Bacteroides	1.80%	0.00%	0.00%
Prevotella	2.70%	0.00%	0.10%
[Paraprevotellaceae];g__YRC22	1.60%	0.00%	0.00%
Planococcaceae;Other	6.40%	1.20%	10.60%
Streptococcus	34.20%	12.20%	10.00%
Clostridiales;f__;g__	0.80%	6.90%	6.30%
Eubacterium	0.50%	3.60%	1.60%
Lachnospiraceae;g__	3.90%	10.20%	14.00%
Butyrivibrio	1.40%	8.40%	6.30%
Roseburia	0.10%	1.80%	0.60%
Succiniclasticum	3.90%	2.30%	8.50%
[Mogibacteriaceae];g__	1.10%	2.00%	3.00%
Anaerococcus	0.10%	0.80%	0.60%
Sporanaerobacter	0.20%	1.60%	0.60%
Bulleidia	0.90%	9.70%	6.90%
Catenibacterium	0.60%	0.20%	1.30%
Desulfovibrio	1.10%	0.00%	0.00%
Succinivibrio	0.10%	0.00%	0.10%
Treponema	1.30%	0.00%	0.00%
Pyramidobacter	1.60%	0.00%	0.10%

Figure 3.8a. Representation(%) of taxonomically assigned microbial OTU reads (Illumina MiSeq) showing treatment effect at the family/genus level on Control, DL & MEEO treatments in the RUSITEC

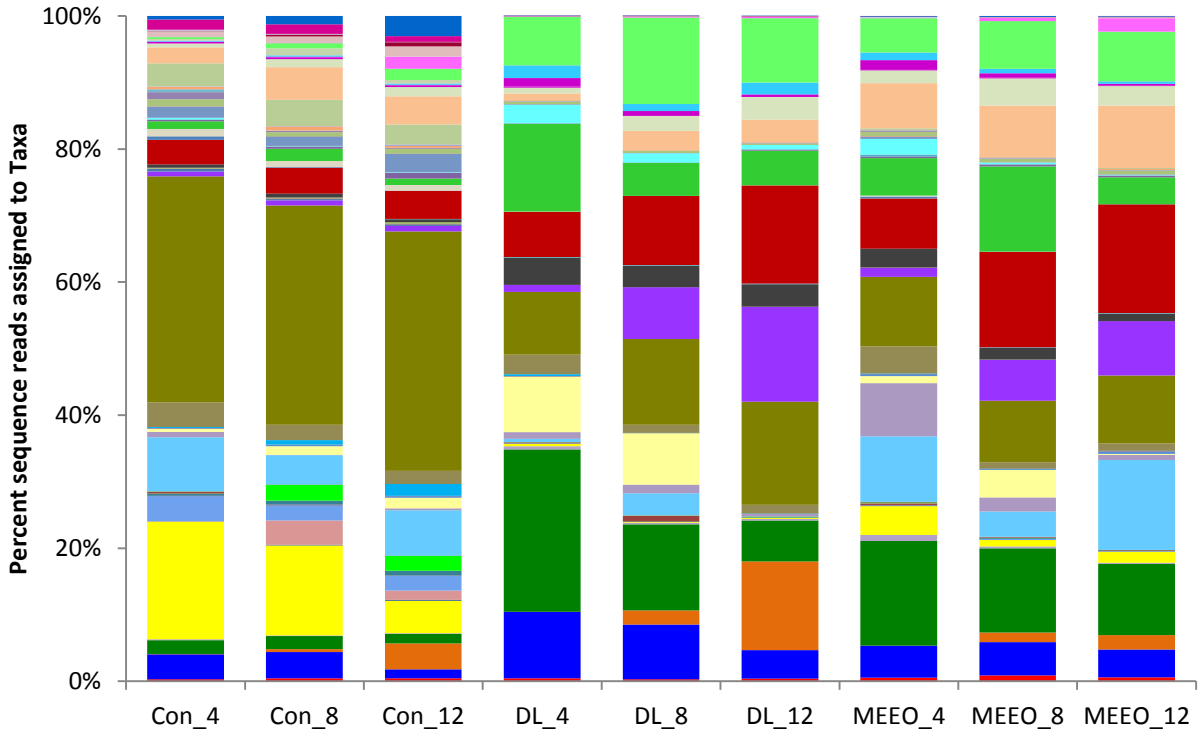


Figure 3.8b. Representation (%) of taxonomically assigned microbial OTU reads (Illumina MiSeq) showing treatment effect at the family/genus level on control, L & MEE treatments in the RUSITEC at different sampling time (D4, D8 & D12). Legends as in Fig 3.8a).

Archaeal OTUs were consistently recovered from the Illumina MiSeq deep sequencing. However, the entire kingdom represented by *Euryarcheota* was represented by methanogens. These sequences represented only 0.4% of all sequence reads in both control and L treatments, whereas their numbers were increased by 50% to 0.6% in MEE treatment (Figure 3.9a). All of the archaeal sequences were represented by one genus, i.e.*Methanobrevibacter*. A detailed analysis of the fermentation days shows that they remained fairly stable all through the 3 time-points while the methanogen numbers increased in the MEE treatments (Figure 3.9b).

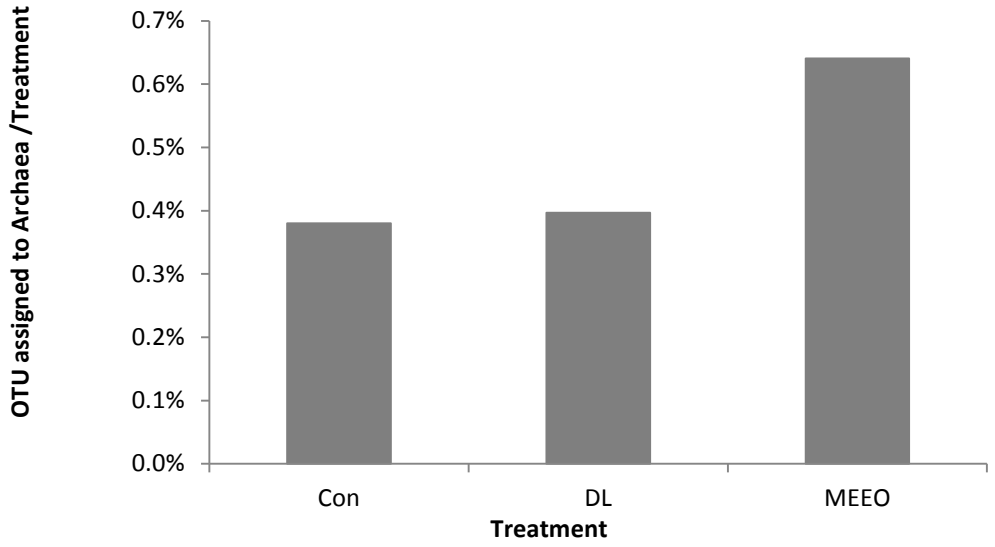


Figure 3.9a. Percentage of taxonomically assigned archaeal OTU reads at the genus level on Control, L & MEEtreatments in RUSITEC. Cumulative result of each treatment shown.

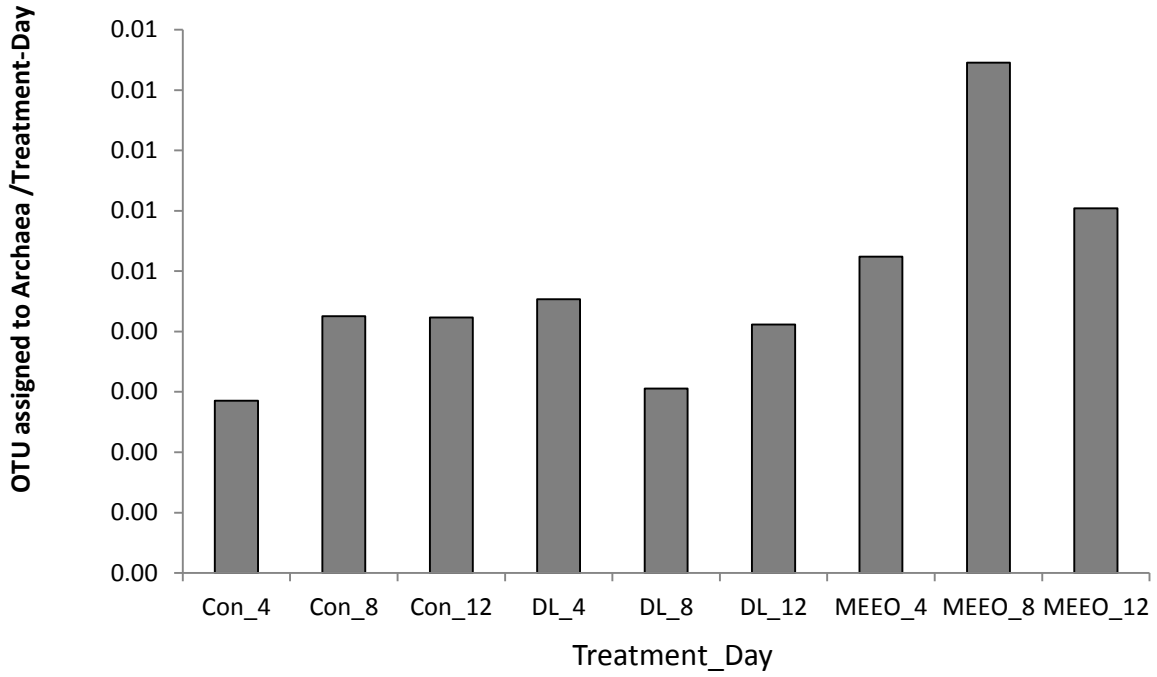


Figure 3.9b. Percentage of taxonomically assigned archaeal (Methanobrevibacter) OTU reads showing time effect at the genus level on Control, L & MEE treatments in RUSITEC at different sampling times (D4, D8 & D12).

Discussion

The results from this study demonstrated that both *M. ericifolia* essential oil and its major compound L can produce a persistent effect on methane and *in vitro* fermentation in an open system. In batch culture and with an analogous dose (500 µL/L, BA2), L reduced methane production by 20% when compared to the control, and a similar reduction was observed here (30% initial, 17-18% final). However, the percentage reduction in methane dropped by 10% in the later two phases, which implies that some adaptation to the compound may have occurred. The reduction in methane observed with *M. ericifolia* was also about 30% compared to control, but only in the initial period, while in the other two periods, methane was similar to the control. This may explain why we did not see a significant reduction in methane production when we tested an analogous dose of *M. erecifolia* in our batch system (625 µL/L, BA1). L caused some small reduction in gas and VFA, but these were transient and it is possible that the microbes eventually adapt. Some care should be taken when considering the use of L, because there have been reports that L may affect animal health at doses above 100 g/kg food, but these studies have only been done in rodents.

We have also confirmed that the major compound in *M. erecifolia* is L, which persisted in the fermentation fluid throughout the experiment and this is consistent with the findings of antimethanogenic effect in the system. Therefore it is likely that L is a major compound in this EO responsible for the effect. As the antimethanogenic effect was more pronounced towards the end of the incubation period may potentially be explained with findings here - while it was still affecting the methanogens, it is possible that the other rumen microbes responsible for the metabolism of L were also affected or adapted over time and hence it was less degraded, therefore more available in the fermentation fluid.

Microbial qPCR analysis provided a molecular insight into selected microbial targets relating to the physiological process of fermentation during fibre-digestion, like gas and methane production and can corroborate physiologic assay results with respect to the chosen target populations in the simulated rumen. *F. succinogenes, R. flavifaciens* and *R. albus* are important fibre-digesting bacteria and were used in qPCR assays (Koike & Kobayashi 2001 and Denman & McSweeney 2006). Our qPCR assays for these bacteria showed either slight increases in the *R. flavifaciens* and *F. succinogenes* population or no significant changes in numbers relative to the controls for both compounds. However, *R. albus* levels were reduced overall; yet, their numbers steadily improved over each time point for both the compounds suggesting recovery of their population, possibly due to adaptation.

The Methyl coenzyme-M reductases (mcrA) gene product is the final enzyme that reduces CH_3-group to CH_4 in the methanogenic pathway. Thus mcrA-qPCR has been used in several studies to track treatment regimes aiming to mitigate methane production in the rumen (Denman et al., 2007; Mosoni et al., 2011). In our assays, the overall methanogen numbers assayed by mcrA qPCR was not significantly different compared to the controls or showed both a slight increase and decrease for the two treatments at different time points. In addition, we designed two additional qPCR primers (RCC1 for rumen cluster-C and Mbr for *Methanobrevibacter* spp.) based on their 16S r RNA gene. The RCC primer showed an overall reduction as well as progressive strong reduction in their numbers across all time points for both treatments. The Mbr-based assay numbers, however, were slightly higher for Me-EOC overall, whereas compound 'L' showed a reduction in numbers for both d15 & d19. These results suggests that the RCC groups could be targeted by these compounds for methane mitigation. This group may be underestimated in many rumen methanogen surveys and may have been overlooked in earlier methane mitigation studies, since they were not tracked or were not targets of molecular studies of ruminal methanogens (Janssen & Kirs 2008; Jeyamalar et al., 2010; Poulsen et al., 2013; Wei et al., 2014).

Deep sequencing provided greater depth into the effect of the treatments on individual or groups of bacteria during fermentation. In general, it appears that the two treatments affected the microbial populations. *Bacteroides*, *Bacteroidales*, *Streptococci, Prevotella, Desulfovibrio*, *Treponema* and

Synergistetes were severely or completely inhibited by both treatments. On the contrary, *Bulleida*, *Butyrivibrio, Clostridiales*, *Lachnospiracea* and *Eubacterium* increased their representative OTUs significantly. The latter two also encompass known members of acetogens which compete for H_2 and CO_2 in the rumen systems against methanogens to convert them into acetate, an important SCFA in animal nutrition. Further confirmation, involving actual enumeration of these bacteria by qPCR is required. *Bifidobacteria* and *Coriobacteriacea* OTUs were also increased considerably by L & MEE treatments. *Bifidobacteria* are involved in starch/glucose degradation, producing acetate and lactate as end products. These changes may explain some increase in acetate observed in the fermentation with these treatments. Surprisingly, members from other groups that are fibre digesters like *Fibrobacter* and *Ruminococcus (F. succinogenes, R. albus and R. flavifaciens)* were not represented in these sequences, although qPCR analysis of the same RUSITEC samples showed that *R. flavifaciens* and *F. succinogenes* numbers were increased in MEE and L treated samples. *Ruminococcacea* OTUs as a group are represented by ~0.8% in the control, 0.34% in L & 0.56% of the sequences in MEE (data not shown), whereas *Fibrobacter* is not represented by any OTUs and the difference may be explained by different sensitivities of the methods.

Among the archaea, only methanogens were represented and only *Methanobrevibacter* genus was found in the treatments. This is the predominant genus represented in the rumen of cattle, sheep, goats, camel and all known enteric methanogens both in terms of predominance in numbers as well as in the spread of species under this genus. For this microbe, the qPCR analysis is in agreement with the current Illumina data, in particular, L that had only marginally higher or a trend towards decreasing *Methanobrevibacter*in both methods. However, there was no representation of either Rumen Cluster C (RCC) group OTUs or *Methanosphaera* sequences that were found using qPCR. The latter is emerging as a well recognised member of the rumen/gut based on deep-sequencing studies with a significant share of the methanogen sequences along with the RCC clade members. It is therefore important to optimize methodology to overcome these discrepancies between the two methods.

Conclusion

In conclusion, the results of the present study confirmed L as a major compound and as a potent antimethogenic compound in *M. ericifolia*. It has persisted in the system when included either as part of essential oil or as a pure compound. These findings provide further evidence that the antimethanogenic effect observed in the RUSITEC may be assigned to L, that the compound is relatively stable in the mixed rumen population and that there may be some adaptation of rumen bacteria, but not methanogens in the system. The mechanism is likely to be a combination of a direct effect on rumen methanogens, in particular RCC clade and promoting alternative hydrogen pathways and microbes responsible for those pathways, such as acetogens, that divert hydrogen from methane.

These results also provide preliminary evidence that L may cause a 20-30% reduction in methane when mixed with a concentrate diet at 16 g/kg food. While it is important now to translate these findings *in vivo* and examine the conditions under which this effect persists (dose, type of diet), these findings highlight the usefulness to introduce RUSITEC testing as a platform for narrowing down treatments for *in vivo* testing.

Implications

L shows promise as a natural bioactive compound, causing up to 30% reduction in methane without a significant reduction in overall fermentation. Further optimization (dose, type of diet) is desirable before developing protocols for testing *in vivo*.

Experiment BA 30c - Optimisation of dose, substrate and application form of L in batch for RUSITEC testing

Aim: to investigate the dose response of L in the *in vitro* batch culture using two types of substrates (fibrous and concentrate) and determine the doses/diet(s) that could be tested further in the RUSITEC.

Background

In our preliminary *in vitro* batch fermentations, we observed that inclusion of L, when dissolved in ethanol and at a concentration of 62.5 µL/g DMi, along with a concentrate-based diet resulted in a significant reduction in methane production, with little inhibition in overall gas (Experiment BA 2). The compound was tested further in the RUSITEC, but with only one dose, one type of substrate, and L was dissolved in ethanol (Experiment BA 3). Subsequent batch tests indicated that it may not be necessary to dissolve the compound in ethanol (Experiment BA 32, data not shown), as the L was equally effective with and without dissolving in ethanol.

Materials and methods

Treatments were prepared and samples collected and analysed using *in vitro* batch fermentation as described in Durmic *et al.* (2010).

A total of 12 treatments, including five levels of 'L': 31.25, 62.5, 100, 125 and 250 µL/g DMi, were combined with two different substrates, fibrous (oaten chaff) and a concentrate (EasyOne, Milne Feeds, Welshpool, Australia; 14.5% CP; ME 11.0 MJ/kg; crude fibre 20.0%). Substrate was added to a tube then filled with buffered rumen fluid. Compound L was dispensed straight to the tubes (3.125, 6.25, 10, 12.5 and 25 µL/tube).

Results

Addition of compound L resulted in a significant reduction in methane at doses of 62.5 µL/g DMi (forage) or 31.25 µL/g DMi (concentrate) and above (Table 3.8). All reductions in methane were associated with a small (i.e <20%) to moderate (i.e. <40%) reduction in total gas production, with reductions in gas below 20% observed with selected doses. There was a dose-response effect with the addition of L, with a strong correlation (R^2 = 0.96 and 0.98) between dose of L and methane, and a similar effect amongst the two substrates evaluated (Figure 3.6).

Table 3.8. Dose-response in antimethanogenic activity of L when added to a fibrous or concentrate substrate and tested *in vitro*.

Treatment (µL/g DMi)	CH_4 (mL/g DMi)		SEM	Gas (mL/g DMi)		SEM	% Reduction Compared to Control	
							CH_4	Gas
Forage								
Control	40.0		0.560	381		0.220		
31.25	39.1		0.680	377		0.270	2	1
62.5	33.6	*	0.560	350	*	0.220	16	8
100	27.5	*	0.560	321	*	0.220	31	16
125	15.7	*	0.560	294	*	0.220	61	23
250	1.1	*	0.680	264	*	0.270	97	31
Concentrate								
Control	44.5		0.521	379		0.153		
31.25	41.8	*	0.521	372	*	0.153	6	2
62.5	37.6	*	0.521	351	*	0.153	15	7
100	29.1	*	0.521	318	*	0.153	35	16
125	21.9	*	0.521	309	*	0.153	51	18
250	1.9	*	0.638	230	*	0.188	96	39

* significantly different than control (P < 0.05)

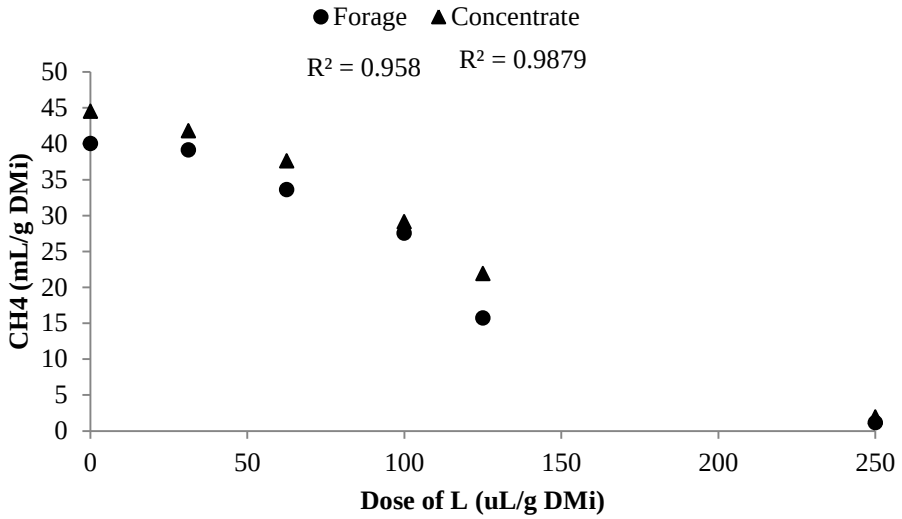


Figure 3.6. Correlation between the dose of L and methane yield

Discussion

Overall, we have confirmed the antimethanogenic effect of compound L when added to either a fibrous or a concentrate substrate. In general, the level of reduction in methane and gas was similar between the diets, except that there was a small but significant reduction (6%) observed with the lowest dose 31.25 µL/g DMiwith the concentrate substrate. In addition, the 125 µL/g DMi dose was

more effective with the forage substrate compared to the concentrate substrate (61% reduction vs 51% reduction). There was a strong correlation between methane production and the dose of L in both substrates. The doses that were effective with both substrates, i.e. inhibited methane by more than 10%, while maintaining gas production (i.e. reduction less than 20%), were 62.5 µL/g DMi, 100 µL/g DMi and 125 µL/g DMi, and we used this information to guide the design of the RUSITEC experiment (next section). Since there were no differences in the effectiveness in L at two of these doses between the two substrates, the subsequent RUSITEC study was focussed on examining whether the higher dose of L (i.e 125 µL/g DMi), compared to the dose tested previously in the Rusitec (i.e 62.5 µL/g DMi), in combination with concentrate substrate reduces methane without affecting fermentation.

Implications

Doses for second testing of L in the RUSITEC have been selected.

Experiment BA 37 - RUSITEC 2. Testing of two EOC – L and C

Background

In our previous studies, we identified several potent EOC that are capable of reducing methane without significantly affecting gas (BA 2). We focused on L and, in preliminary studies in the RUSITEC, confirmed that the dose of 62.5 µL/g DMi (500 µL/L fermentation fluid) reduced methane production by 17%. In more recent batch studies, we have shown that another EOC, C, could be even more effective than L, causing the same effect on methane with only half of the dose of that required for L.

The aim of the current study was to examine the effect of two doses of L and C. We selected the doses that caused 15% and 50% reduction in methane in batch, to test in the RUSITEC: effectively 62.5 µL/g DMi and 125 µL/g DMi for L and 31.25 µL/g DMi and 62.5 µL/g DMi for 'C'. Lower doses of L were not effective in the batch, while higher doses for L and C caused a reduction in total gas > 20%.

Materials and methods

EOC and RUSITEC conditions were similar to that reported earlier in RUSITEC 1 (BA 3), with the exception that EOC were included at two different levels and without dissolving in ethanol. The doses were selected based on their effect in batch IVFT and RUSITEC 1. To account for the difference in the ratios between incubation fluid volume and dry matter incubated (i.e. there is 10 g/L in batch and 25 g/L in the RUSITEC), the doses from the batch were divided by 2.5 to obtain the dose required for the RUSITEC. Effectively this means that the doses for L in the RUSITEC were 25 µL/g DMi and 50 µL/g DMi (corresponding to 62.5 and 125 µL/g DMi in batch), and for C 12.5 µL/g DMi and 25 µL/g DMi (corresponding to 31.25 and 62.5 µL/g DMi in batch).

Results

When compared to the control, the 'medium' level of C and L (i.e. 25 µL/g DMi) and 'high' level of L (50 µL/g DMi) resulted in a reduction in methane (85%, 55% and 84%, respectively, Figure 3.7; $P<0.05$). The effect persisted over 14 days of incubation (Figure 3.8).

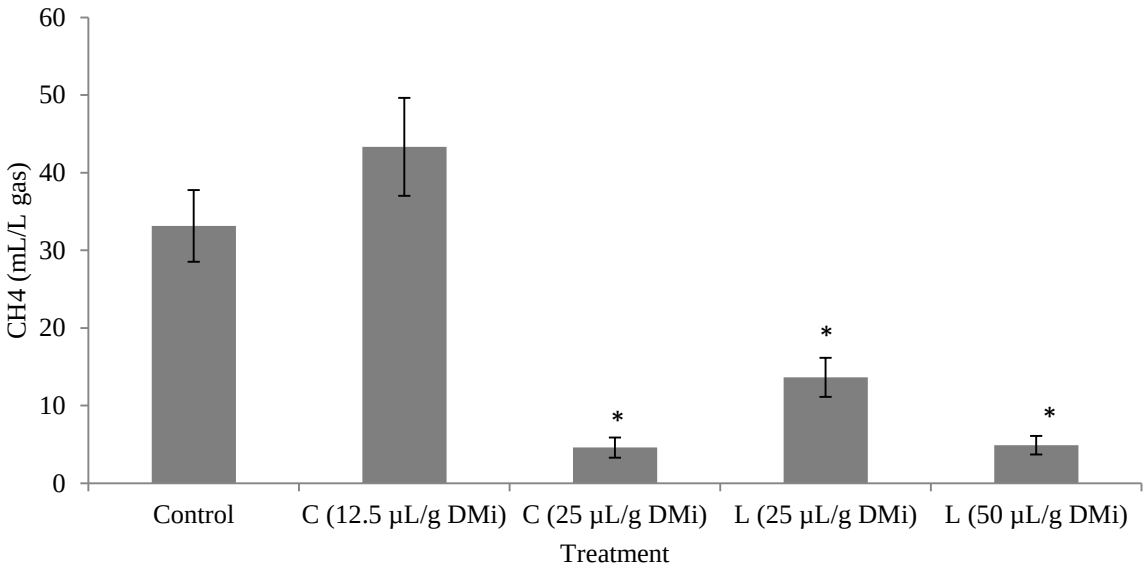


Figure 3.7. Average methane production from treatments, including two levels of C and L tested in RUSITEC.

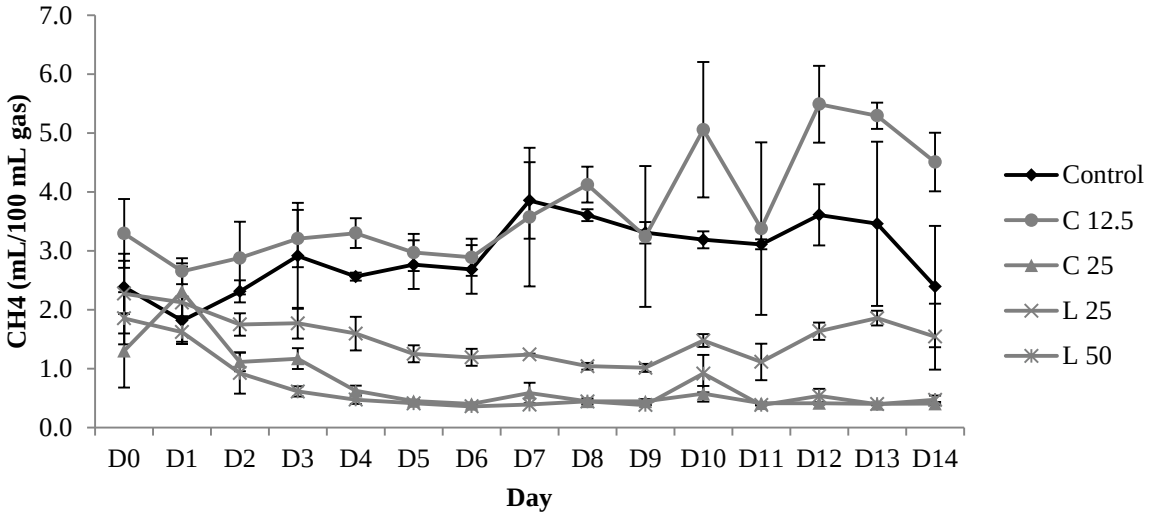


Figure 3.8. Average daily methane concentration in overall gas collected over 24 h from fermenters for 14 days.

Average total gas produced was not affected by any of the treatments when compared to the control (Table 3.9). However, total VFA, acetate, branched chain fatty acids (BCFA) and NH_3 concentrations were reduced with all EOC treatments ($P<0.05$), excluding 'C' 12.5 µL/g DMi. Propionate was reduced with all EOC, while butyrate was reduced only with 'L'. Compared to the control, the reduction ranged between 30-40%, depending on the treatment. Similar level of reduction was observed in the NH_3 concentrations.

The total VFA were also reduced in all EOC treatments over the time, reaching the lowest values at the last days of sampling (Figure 3.9). The largest drop i.e. 40% between day 0 and day 12 was observed in L 50.

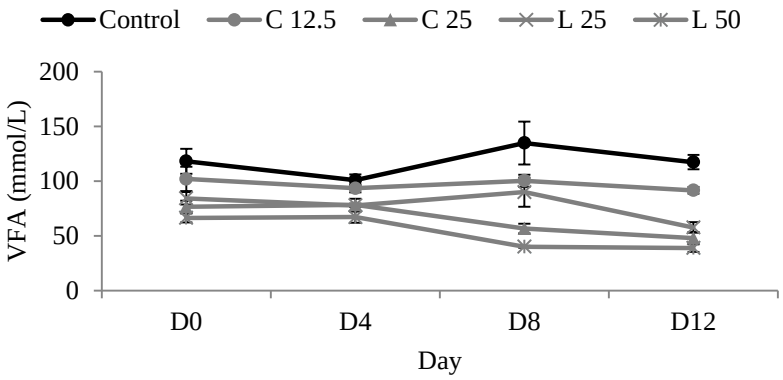


Figure 3.9. Total VFA concentrations (mean±SEM) on day 0, 4, 8 and 12

Table 3.9. Average daily fermentation parameters during fermentation of two doses of EOC, C and L with a concentrate base substrate in the RUSITEC. Significance: within the row, values not sharing the same letter differ (P<0.05)

Treatment	Control		C (12.5 µL/g DMi)		C (25 µL/g DMi)		L (25 µL/g DMi)		L (50 µL/g DMi)	
Gas produced (mL/day)	720		743		756		728		694	
Total VFA (mmol/L)	118	a	102	ab	76	c	66	c	84	bc
Acetate (mmol/L)	68	a	56	ab	45	bc	40	c	45	bc
Propionate (mmol/L)	27	a	14	b	9	b	7	b	9	b
Butyrate (mmol/L)	13	c	20	a	12	c	9	d	17	b
BCFA (mmol/L)	4.5	a	4.0	a	2.2	b	1.6	b	2.4	b
NH3 (mg/L)	452	a	397	ab	312	bc	283	c	332	bc

Discussion

The results from the current study suggest that both pure compounds, L and C, at selected doses, can reduce methane production significantly from fermentation of a concentrate-based diet in the RUSITEC. The effect persisted for 14 days and the effective reduction (i.e. over 70%) was observed with as little as 25 µL of C per g DMi. This treatment also produced the highest amount of gas. Interestingly, the two doses of C tested here resulted in contrasting activities, and C was ineffective at the lower dose.

Overall microbial activity, as judged by VFA and NH_3 was reduced when EOC were included at dose of 25 µL/g DMi and above. As these doses were the only ones also found effective in methane reduction, it would be beneficial to examine a range of doses between the effective and non effective one to optimise a feasible application that is still effective *in vivo*. As the effect on fermentation appear to be lower than the effect on methane, it may be possible to find a dose that is still effective on methane and having minimal effect on overall microbial activity.

Essential oils and compounds are a focus of research to find potential feed additives for livestock because they are aromatic and have been reported to promote appetite, intake and can have positive effects on digestion and milk production (Varga et al 2004, Meyer et al. 2007). They also have some potent antibacterial properties against animal gut pathogens (Sims et al. 2004, Durmic et al. 2008, 2014) and, therefore, may have multiple potential benefits in the animal - boost production and control diseases in animals while mitigating methane. Further studies to confirm the effect on methane *in vivo* may warrant progressing these towards developing a new additive. Further investigation of other EOC that were equally effective in the preliminary batch testing (Experiment BA 2) may diversify the spectrum of compounds that could be valuable for this application.

Implications

Several combinations of EOC were effective at reducing methane substantially (i.e. >70%) over two weeks, but they were also affecting fermentation in the RUSITEC. These combinations need to be optimised further before being progressed and tested *in vivo*.

4. Testing of fractions and pure compounds against methanogens in pure culture

Background

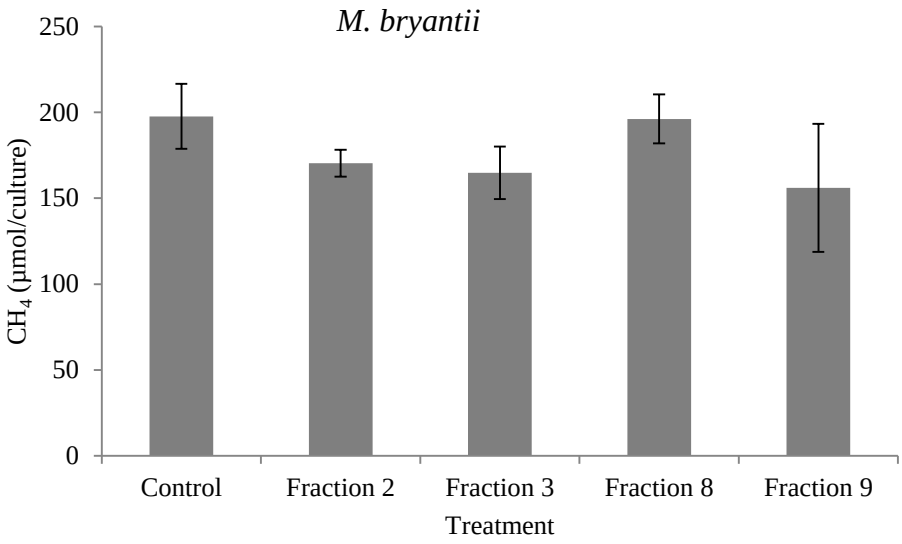
Our previous experiments narrowed down some candidates for testing against methanogens in pure culture. We have identified several potent antimethanogenic fractions from biserrula. Concurrently, we conducted testing of pure plant compounds (essential oil compounds) that were available commercially, in adequate purity and quantities (in particular for RUSITEC) for testing. Amongst these, L and C were of the most interest. We opted to progress these two diverse types of candidates i.e. fractions (mix of compounds) vs pure compound for two reasons. As the PSCs in biserrula are still unknown, we adopted an approach to test a broader mix of compounds, present in selected active fractions, as the representatives of the variety of PSCs in this plant to maximise the possibility of finding the active ones. We also wanted to examine if the mix of compounds would have more diverse effects opposed to a single compound.

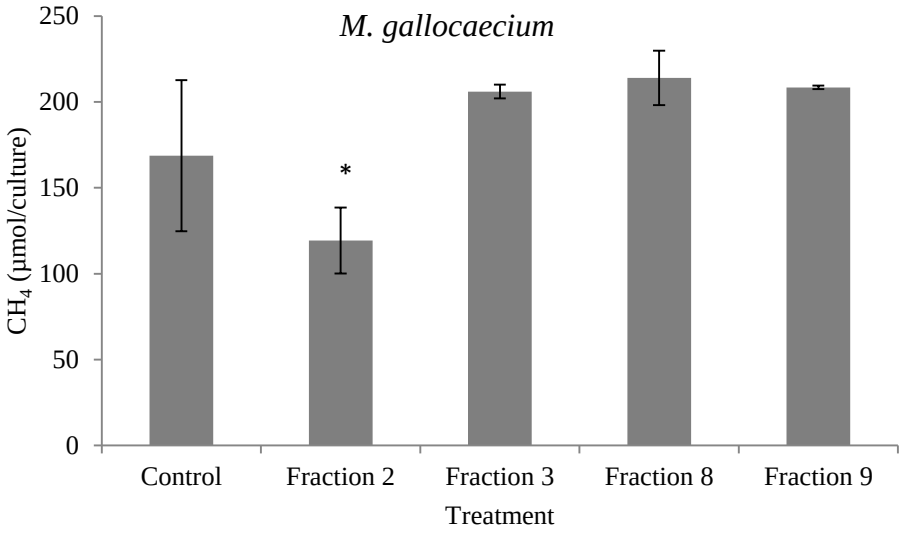
Experiment BB 4 - The activity of selected fractions from biserrula against methanogens in pure culture

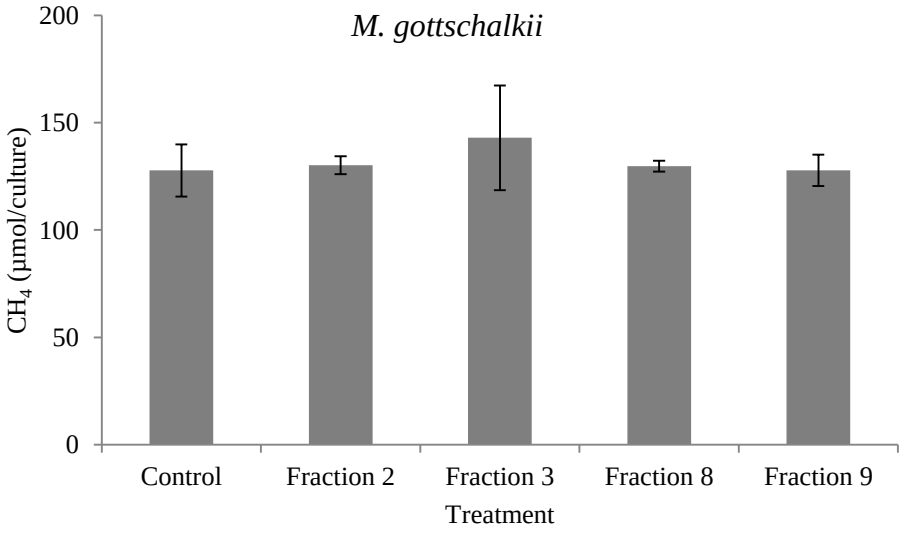
Aim:to examine if selected bioactive fractions will have an effect on key types of methanogens and the type of effect (static vs cidal).

Results

All four fractions of biserrula showed significant reduction in methane production by *M. stadtmanea,* except fraction 8, while in *M. ruminantium* and *M. gallocaecium* cultures, fraction 2 was active (Figure 4.1). *M. bryantii* and*M. gottschalkii*were not affected by any of the biserrula fractions tested.


M. bryantii
250
200
150
100
50
0
CH4 (µmol/culture)
Control
Fraction 2
Fraction 3
Fraction 8
Fraction 9
Treatment


M. gallocaecium
250
200
150
100
50
0
CH4 (µmol/culture)
*
Control
Fraction 2
Fraction 3
Fraction 8
Fraction 9
Treatment


M. gottschalkii
200
150
100
50
0
CH4 (µmol/culture)
Control
Fraction 2
Fraction 3
Fraction 8
Fraction 9
Treatment

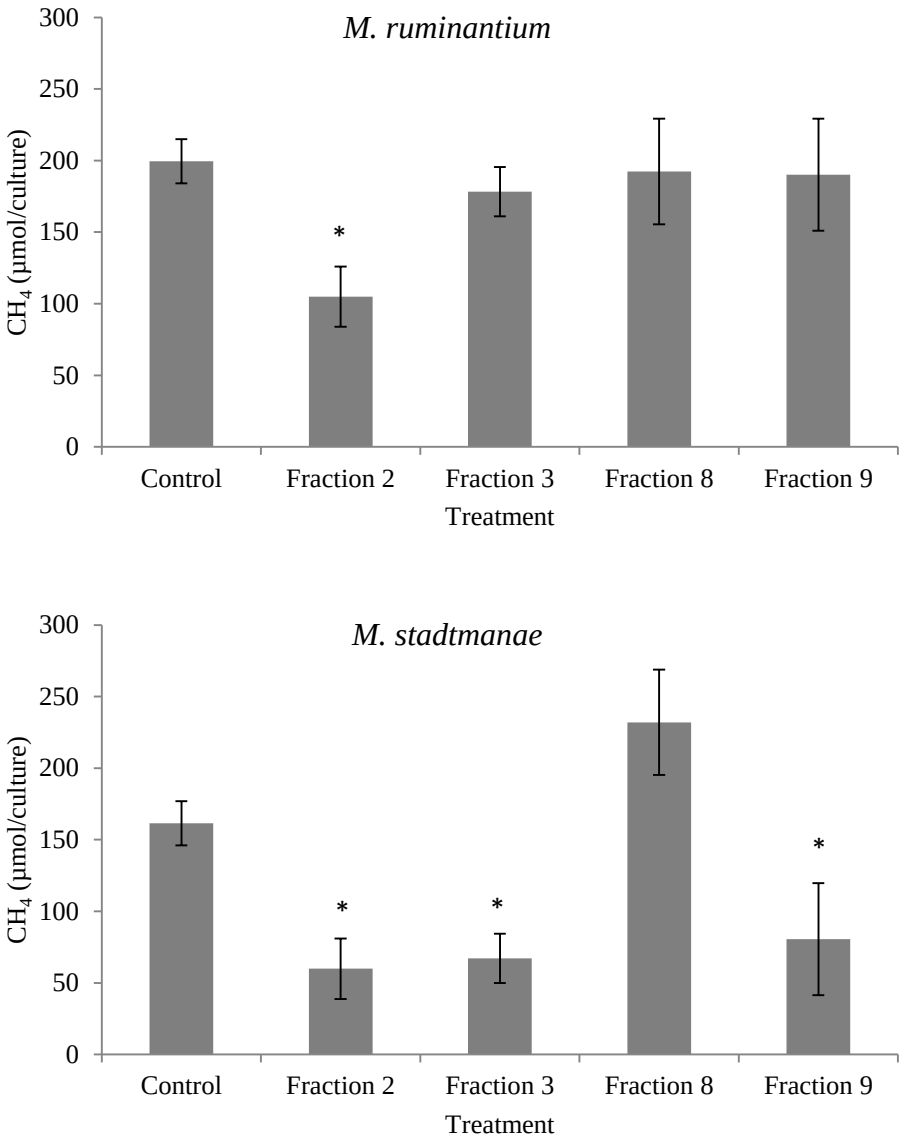
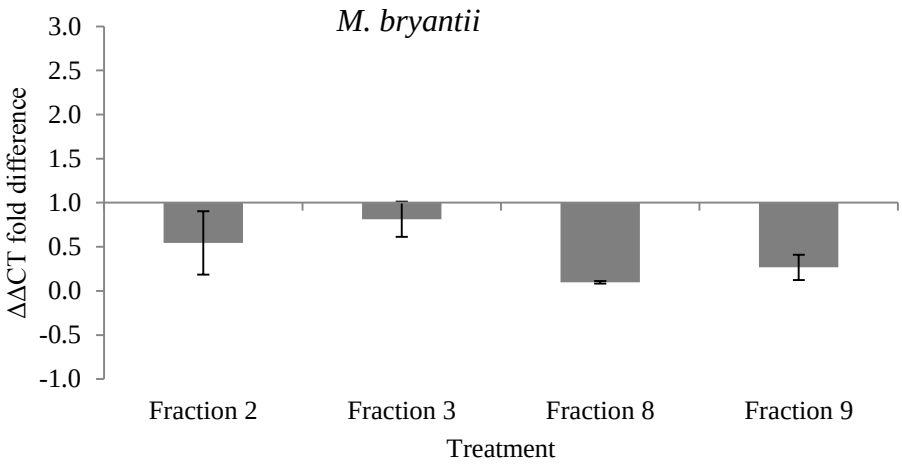
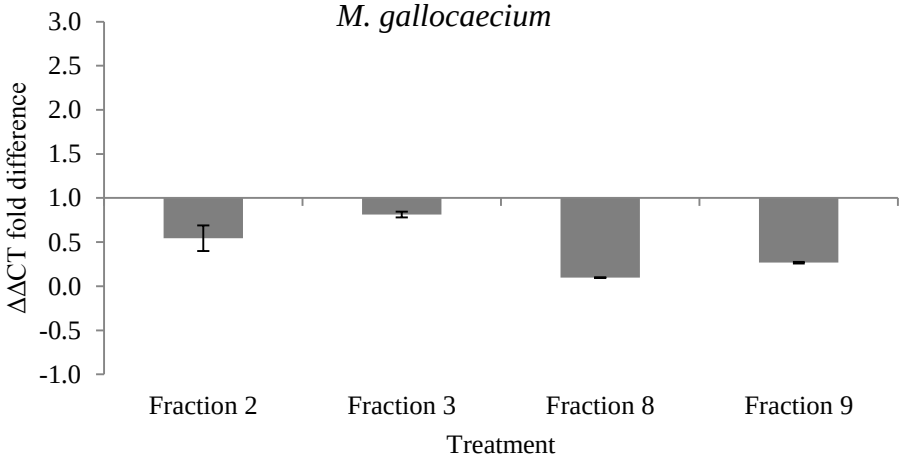
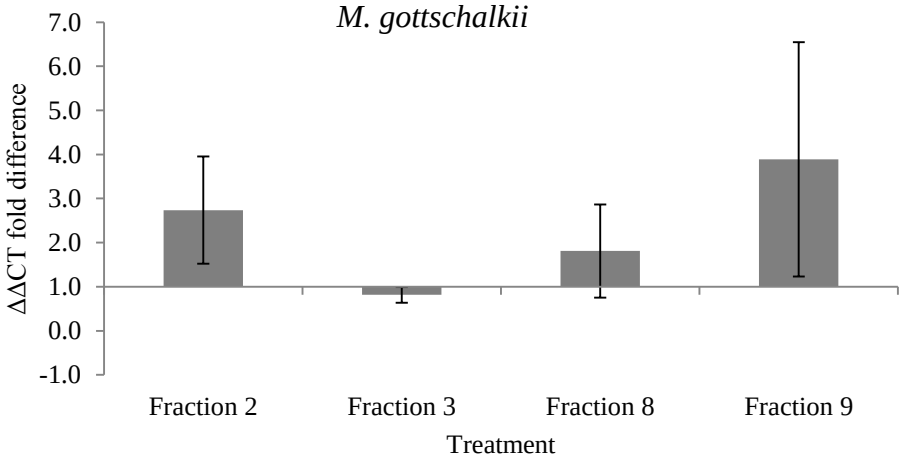


Figure 4.1. Methane concentration in headspace (mean ± SEM) in cultures of selected methanogens when exposed to different fractions of biserrula. Control - methanogen grown in presence of EtOH. * - significantly lower than control

As judged by lower ΔΔCT fold differences compared with corresponding controls (i.e. ΔΔCT =1.0 in corresponding control treatments), all the fractions of biserrula tested showed a reduction in the microbial cell growth of *M. bryantii, M. gallocaecium* and *M. ruminantium,* while in *M. stadtmanae,* and *M. gottschalkii* only selected fractions were inhibitory(Figure 4.2). Fraction 3 was inhibitory to all five methanogens.


M. bryantii
ΔΔCT fold difference
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
Fraction 2
Fraction 3
Fraction 8
Fraction 9
Treatment


M. gallocaecium
ΔΔCT fold difference
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
Fraction 2
Fraction 3
Fraction 8
Fraction 9
Treatment


M. gottschalkii
ΔΔCT fold difference
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
Fraction 2
Fraction 3
Fraction 8
Fraction 9
Treatment

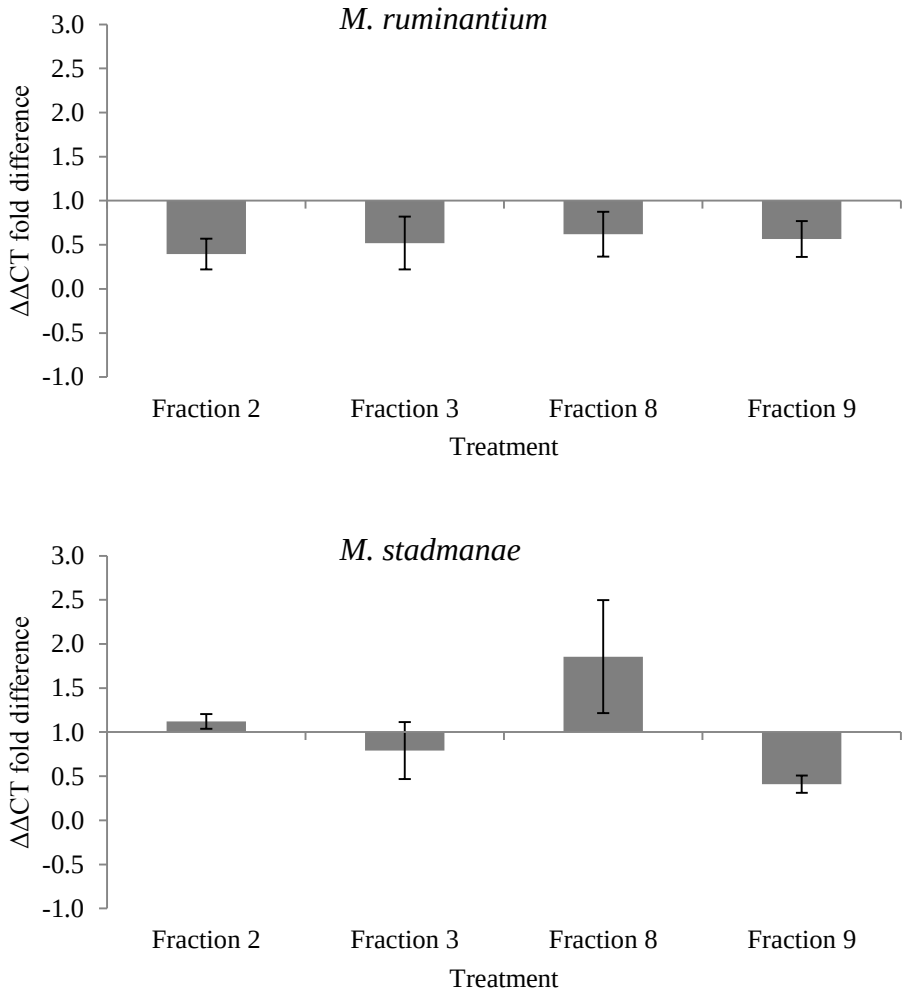


Figure 4.2. Microbial cell growth (mean ± SEM) as estimated by cell gene expression, ΔΔCT fold difference compared to control) of methanogenic pure cultures exposed to biserrula fractions. Negative values indicate inhibition.

Based on their effect in pure culture, fraction 2, 3 and 9 were selected for further testing in static/cidal manner. None of the fractions showed a total inhibition (cidal activity) on the pure cultures of methanogens, as methane was detected in the headspace of all the treatments (Figure 4.3). However, fraction 2 showed a strong inhibition/equivocal cidal effect (i.e. methane < 70 µmol), but only on *M. stadtmanae*, *M. bryantii* and *M. gallocaecium.* Other active fractions showed a static effect (i.e. methane > 70 µmol).

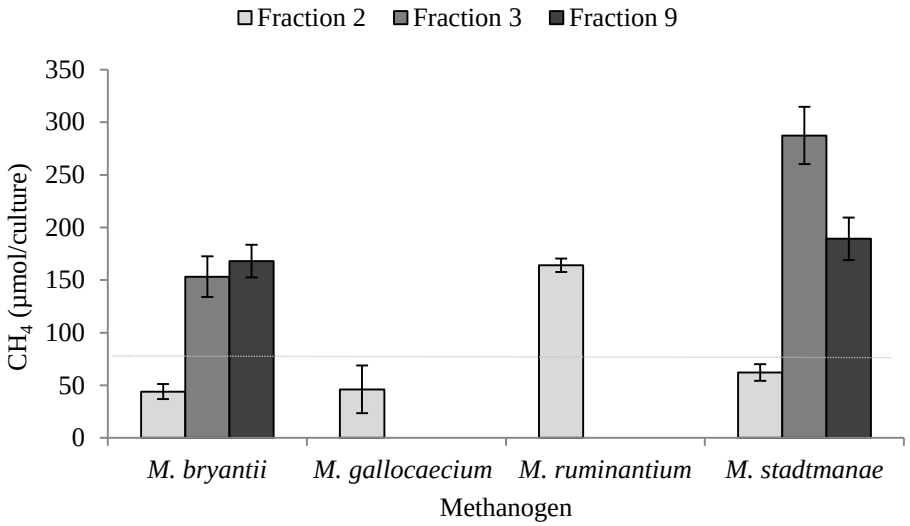


Figure 4.3. Methane concentrations in headspace (mean ± SEM) from cultures of methanogens sub-cultured in fresh media after being exposed to fractions 2, 3 and 9. Line crossing at 70 µmol distinguishes static (above the line) and equivocal cidal (below the line) effect

There were only selected treatments within each fraction that simultaneously reduced methane and microbial cell growth, with only fraction 2 also being effective in producing equivocal cidal effect in these (Table 4.1).

Table 4.1. Summary of the inhibitory effects on methane production, microbial cell growth and type of effect (where tested) in pure cultures of methanogens exposed to selected fractions of biserrula. e/cidal - equivocal cidal effect

Methanogen	Fraction			
	2	3	8	9
M. bryantii	growth (e/cidal)	growth (static)	growth	growth (static)
M. gallocaecium	methane/ growth (e/cidal)	growth	growth	growth
M. gottschalkii	none	growth	none	none
M. ruminantium	methane/growth	growth	growth	growth
M. stadtmanae	methane/ growth (e/cidal)	methane/growth (static)	none	methane/ growth (static)

Experiment CSIRO EO/EOC -The activity of selected essential oils (EO) and EO compounds (EOC) against methanogens in pure culture

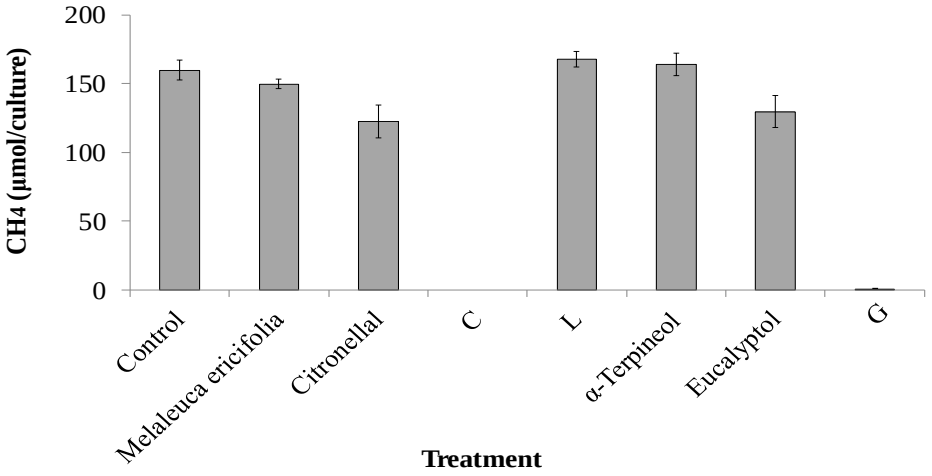
Aim: to examine if selected EO/EOC will have an effect on key types of methanogens and the type of effect (static vs cidal).

Results

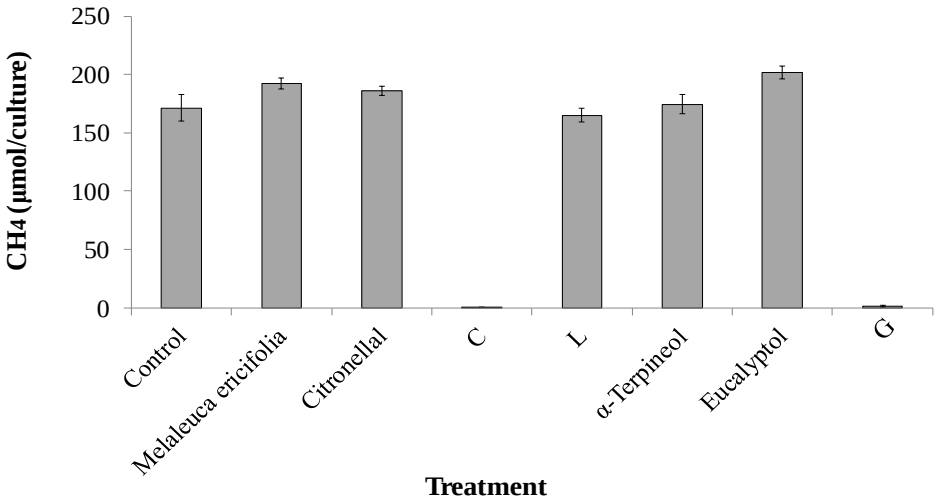
Initial testing of EO and EOC at concentration of 2 µl/ml media, a dose showing reliable inhibition in RUSITEC, with pure cultures*M. smithii* and *M. stadtmanae* showed complete abrogation of growth by all compounds tested (data not shown). Subsequently, a lower concentration, i.e. 6.25 µl/ml was chosen in further tests. At this dose, C and G completely inhibited growth of all methanogens tested

(Figure 4.4). Citronellal was inhibitory to all except *M. gottschalkii*, while L was only affecting *M. bryantii*. Other compounds were not effective against methanogens or showed only a marginal effect.

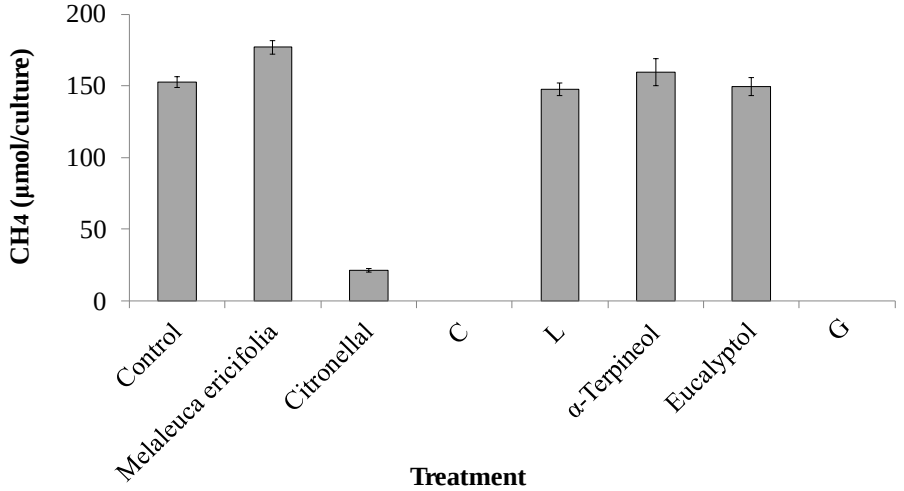
A



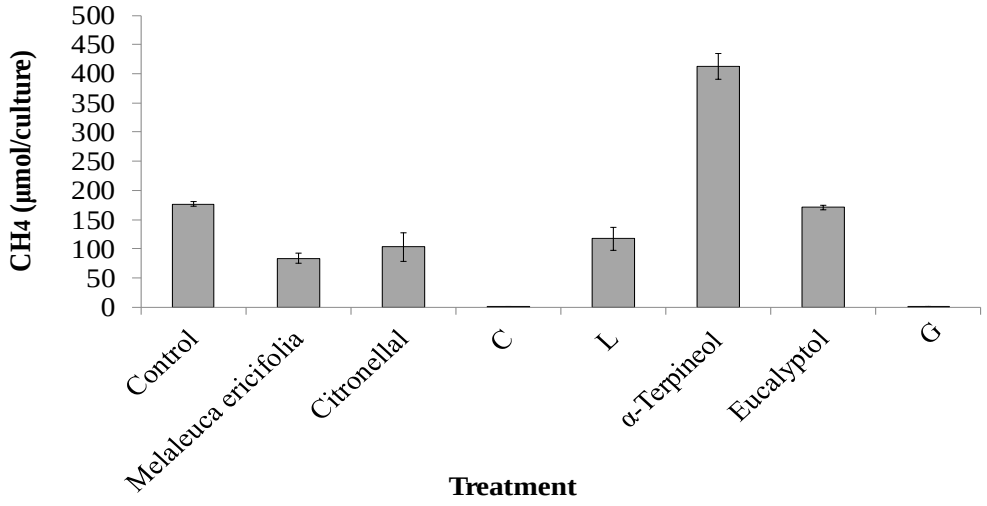
B



C



D



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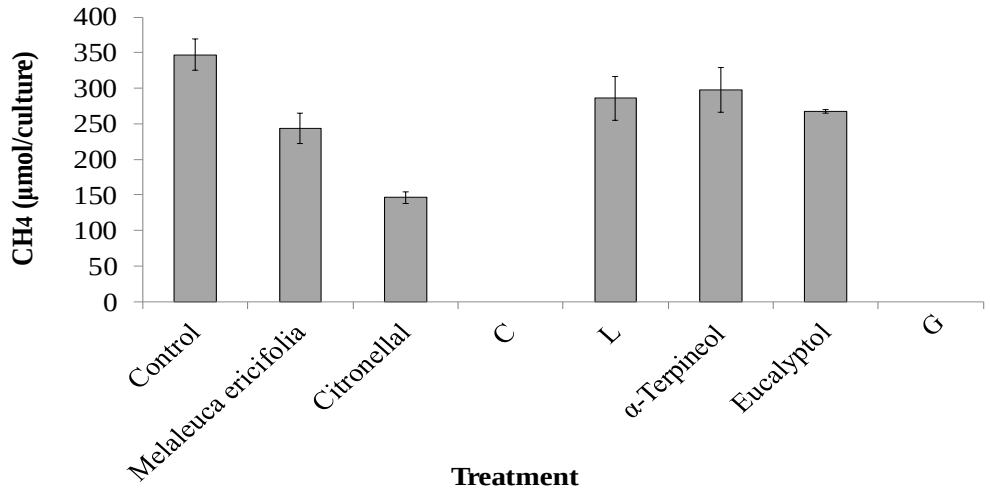

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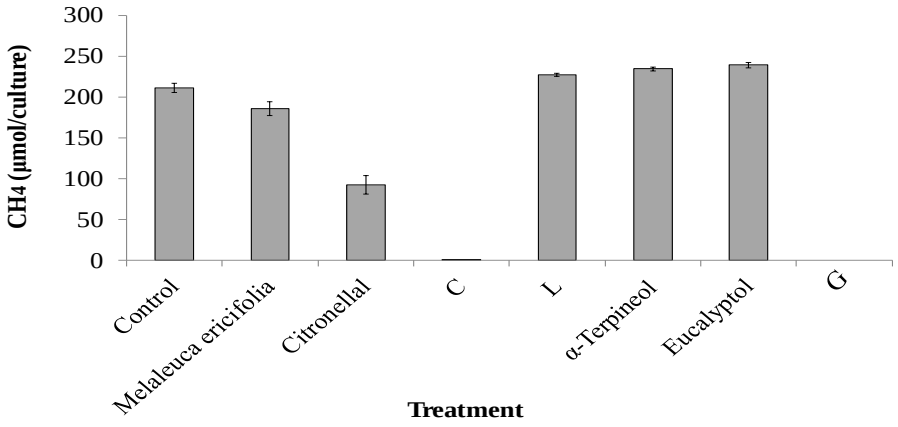

Figure 4.4**.** Methane produced (mean ± SEM) in cultures of selected methanogens treated with different essential oils or essential oil compound (0.63 µl/ml of media).

a:*Methanobrevibacterruminantium*,**b:***Methanobrevibactergottschalkii,* **c:***Methanobrevibactersmithii*, **d:***Methanobacteriumbryantii*, **e:***Methanosphaerastadtmanae*, **f:***Methanoplasmagallocaecium.*

When tested in a dose-response manner, *M. bryantii* appeared to be the most sensitive, reacting to all 4 compounds tested. For example, as little as 0.25 µl/ml of C was required to completely inhibit this methanogen (Table 4.3). Complete inhibition of all three methanogens was achieved with C with 0.25 µl/ml and above, while in *M. erecifiolia* and L at 1.5 µl/ml and above.

Table 4.3. Methane produced (mean ± SEM) in cultures of selected pure methanogens treated with different doses of essential oil or essential oil compounds. *Treatments selected for static/cidal testing

Dose (ul/ml)	*M. gottschalkii*		*M. bryantii*		*M. stadtmanae*	
	CH_4 (umol/ml)	SEM	CH_4 (umol/ml)	SEM	CH_4 (umol/ml)	SEM
			M. ericifolia			
0.00	164	7.1	186	6.2	200	8.1
0.75	174	5.0	2	1.9	192	9.4
1.50	0	0.0	0	0.0	204	4.6
2.00	0	0.0	0	0.3	0	0.0
			C			
0.00	164	7.1	186	6.2	200	8.1
0.13	166	3.6	11	0.7	203	3.7
0.25	0	0.0	0	0.0	0	0.0
0.50	0	0.0	0	0.0	0	0.0
			L			
0.00	164	7.1	186	6.2	200	8.1
0.75	175	5.2	1	0.3	201	5.5
1.50	0	0.0	0	0.0	0	0.0
2.00	2	1.6	0	0.0	0	0.0
			G			
0.00	164	7.1	186	6.2	200	8.1
0.13	161	3.6	176	11.5	175	3.9
0.25	166	2.7	55	10.8	183	1.1
0.50	161	12.6	0	0.4	187	1.4

The treatments that methane inhibition in the dose response experiments were examined further in static/cidal test. When inoculated in fresh media without compounds, treatments from L and *M. erecifolia*in *M. gottschalkii* subsequently grew and produced methane. In contrast, cultures originally treated with C did not grow or produce methane upon dilution, indicating cidal characteristics. For *M. stadtmanae*, L and *M. erecifolia* showed growth and methanogenesis upon dilution. This denotes likely static rather than cidal effect for these two compounds in this species. In contrast, cultures of *M. stadtamanae* treated with C at concentrations >0.25 µl/ml showed probable cidal effect, as they did not exhibit growth or methane production even upon dilution into fresh media. Finally, *M. bryantii* cultures that showed antimethanogenic properties at primary doses (G at 0.5 µl/ml, C>0.25 µl/ml, and *M. erecifolia* and L>0.75 ul/ml) did not grow or produce methane once transferred. This suggests a cidal effect and possible greater susceptibility of this clade to at the least therapeutic doses of these four treatments.

Discussion

The results from this study confirmed that the bioactive fractions of biserrula have inhibitory effects on the key methanogens in the rumen, and to our knowledge, this is the first report on plant fractions affecting methanogens in pure culture. This experiment also showed that the antimethanogenic bioactivity of biserrula fractions varied amongst different methanogens. In terms of methane production, while *M. stadtmanae* appeared to be the most sensitive, responding to three fractions, *M. ruminantium* and *M. gallocaecium* were affected by only one fraction (fraction 2), and *M. bryantii and M. gottschalkii*were not affected with any of the fractions tested. *M. stadtmanae* differs from the others because it canutilise methanol as a substrate to produce methane, and it is possible that the biserrula fractions are targeting these pathways in particular.

Despitea lack of significant differences in methane production, in terms of microbial cell growth, *M. bryantii, M. gallocaecium* and *M. ruminantium* were the most sensitive, being affected by all four fractions. *M. stadtmanae* was sensitive to three fractions, while *M. gottschalkii* was affected by one fraction only.

These effects varied between the fractions, as different fractions seemed to target different types of methanogen and in a different way. There was no fraction that affected all types of methanogens. Fraction 2, however, appeared to produced the most consistent effect, reducing both methane and growth in the majority of treatments, also producing a near-cidal effect. This aligns well with the findings described previously with IVFT and may explain why this fraction was the most potent.

The methanogens also varied in their response. It appears that *M. bryantii, M. gallocaecium* and *M. ruminantium* were the most sensitive, responding in one way or another to the addition of all four fractions. These methanogensare amongst the predominant methanogenic species in the rumen and their sensitivity in pure culture supports our findings of the potency of these fractions or the biserrula plant in mixed rumen batch testing.

In this study, we assesed the antimethanogenic effect of fractions using three approaches - measuring methane, microbial cell growth and recovery of cells after exposure to fractions. It is interesting that these three gave somewhat different results. For example, fractions 3 and 9 predominantly caused a reduction in microbial cell growth, but this was coupled with reduction in methane in only two instances. Fraction 3,which was inactive in the IVFT, was found to be relatively potent in pure culture, inhibiting cell growth amongst four methanogens, while for fraction 8 the opposite occurred. Some of these differences may be explained by the variation in the methodologies and their limitations. While IVFT is a good screening tool and provides a quick and easy tool for narrowing down the treatments to be tested further, it is focused on microbial activity and may not detect reductions in microbial cell numbers. While it is assumed that reduced microbial cell growth will result in decreased overall activity, it appears that some variation may occur. It is also important to understand the limitations of molecular methods applied here. The microbial cell growth was assesed based on numbers of gene copies, which does not take into account cell activity. Cells may still be viable, but slowed down or dormant. This means that even inactive cells may be counted, which may explain why observed a lack of response in methane with some treatments that apparently affected microbial cell growth. This is of particular interest for practical applications, as these dormant cells may in fact regain their activity after the bioactive compound is removed and be a cause of subsequent inefficiency of the method.

It is interesting that a fraction defined as 'inactive' in the IVFT (fraction 3) was actually active against three methanogens in pure culture, and other fractions that were highly active in the IVFT (fraction 2 and 8), did not affect all methanogens, in particular *M. gottschalkii*. While both testing systems we used are artificial, IVFT represents the microbial diversity and at numbers that are more representative of the numeric proportions that they would be in the rumen of the animal whereas when testing in pure culture microbial the cells are present as the same, constant, number across all

species screened. As a consequence, microbes that are normally present in low numbers in the rumen would be articifically higher in pure culture and potentially not affected when present in larger numbers and with no competition. The rumen microbial profiling in sheep revealed a great diversity - for example, one study showed that sheep on pasture in Western Australia were dominated by members of the *M. gottschalkii*, while sheep in Queensland, had those related to *M. gallocaecium* and had only a few members of the *M. gottschalkii* clade. This is of particular importance when developing a methodology, as it appears that there may not be a 'blanket' approach for all and that a mix of compound (or even a whole plant) rather than a single compound may have better prospects. Further, the fraction assesed as active in pure culture but inactive in IVFT, may in fact be degraded by rumen microbes. Hence, in a pure culture, away from degradation, its activity is maintained, but it is inactive in a mixed rumen fermentation system. As a consequence, this fraction *in vivo* would also be ineffective. Overall, it appears important that testing is conducted at both levels to improve the chances of finding and confirming the bioactivity, but it is imperative to take these tests further and confirm them *in vivo*.

While the active fractions tested in the current study were still a mix of compounds, despite their complexity, they have demonstrated a diverse, and in some cases rather specific, effects on different methanogens. Consistent with IVFT findings, fraction 2 appears to be the most effective in pure cultures of methanogens, reducing methane productionand affecting cell growth in several key methanogen species. It was also the only fraction causing equivocal cidal effect and it appear to have a stable effect in a mix rumen population.

When developing a microbial-controlling additive, it is important to achieve persistent and cidal effect. Many attempts in the past have failed for this reason, including the use of antibiotics, as they leave behind viable cells that eventually lead to resistance in microbes. In the current study, none of the fractions tested was lethal to the methanogens, and when the methanogens were removed from a broth containing active fractions, the cultures grewback and the methane-producing activity returned to normal. However, a concentration-dependent microbial growth was observed with other antimethanogenic agents such as bromochloromethane and chloroform (Denman et al., 2007; Knight et al., 2011), and it is reasonable to expect that with increased concentrations, cidal effects may occur with biserrula fractions.

The selected EO and EOC tested have also been shown to be effective in pure cultures of methanogens. The most effective compounds were C, L and G, with C being effective across methanogens tested and at lowest concentrations. Interestingly, *M. erecifolia* had a similar activity to that of L (a pure compound) in a dose-response assay, which provides further evidence that L may be a major compound in this EO responsible for the antimethanogenic effect. Consistent with our findings in the RUSITEC, C was more effective and acted at lower doses. We have confirmed that these two compounds act directly on members of rumen methanogens. Two other candidates, namely G and citronellal also showed some activity, and explain their effect when tested earlier in the mixed rumen population, while eucalyptol was ineffective in both systems.

As expected, the activity of some pure compounds (EOC) were more pronounced than that of mixed compounds (i.e. biserrula fractions) although it may not be a direct comparison, as the concentrations may have been different. However, it is interesting that when a purified compound L found in *M. erecifolia* was tested in a dose-response manner, it did not render any higher activity than the original EO *M. erecifolia.* It is therefore important to know the exact components in the mix, as well as the interactions between these and the role of minor compounds. Some of the EOC produced a cidal effect

In conclusion, the results from the current study demonstrated that biserrula acts through its extractable compounds, same as EOC,making the rumen environment unfavourable for the growth of methanogens, however, the effect depends on both the type of plant fraction/compound and methanogen. Further studies are needed to explore in more detail the actual reason behind low

methane production when methanogens are grown in the presence of selected fractions from biserrula. As fractions contain a combination of PSCs, it would be worthwhile separating them into individual PSCs testing them in pure culture to confirm our results. Moreover, concentration is likely to be a key factor for the effect and dose-response studies are required to find an optimal effective dose. Overall, the results in the current study warrant further investigation of all of these fractions, with particular emphasis on fraction 2 and isolating pure antimethanogenic compounds from biserrula. We have also identified some potent pure compounds found in essential oils/native shrubs in Australia that are ready should be taken forward towards *in vivo* testing.

Implications

The results from this study confirmed that biserrula acts through its extractable compounds affecting key rumen methanogens, and so do some EOC, however the effect will vary and depend on both type of plant fraction/EOC and methanogen. There is not a single fraction from biserrula that targeted all methanogens, and a mix of compounds, once defined, may in fact have better prospects than a single compound. Amongst EOC, the two compounds had a very potent effect and further progress on these may be warranted.

5. *In vitro* testing of other bioactive plants

Overview

The aim of this experiment within the project was to examine several plants that had moderating effects on methane from our previous or current projects. The objective was to examine further the type of compounds that may be involved in antimethanogenic properties in these plants.

Background

Five plants that are identified with the potential to moderately inhibit methane are *Kennedia prorepens* (Durmic et al., 2010), *Calliandra calothyrsus, Leucaena leucocephala* (Durmic et al. 2012), *Dorynicium hirsutum* and *Cichorium intybus* (unpublished data). *Leucaena leucocephala* has been identified asmoderately antimethanogenic, but of interest for tropical Australia and complementing the work in B.CCH.6510 (role of tannins and other compounds in leucaena). Mimosine, condensed tanins and flavanol glycosides are main compounds in this plant, but reports on their role in reducing methane are contradicting. While Tan et al.(2011) found leucaena tannins as potent methane inhibitors *in vitro*,Soltan et al.(2013) implied potential existence of constituents other than these two in leucaena that may be responsible for this effect. *Cichorium intybus* isa moderately antimethanogenic plant (B. CCH 1012, B. CCH 6540). Low methane yield was found in some *in vivo*studies (Swainson et al., 2008) and not in others (Sun et al., 2011). There are several reports on the presence of tannins in this plant and activity against gastrointestinal worms (Heckendorn et al., 2007), while original accessions (i.e. Puna) may contain lactucin and lactone that have some medicinal uses. *Dorycnium hirstum* is another plant under investigation in B. CCH 6540 and found to bemoderately antimethanogenic (unpublished data) and other species of this plant are reported to contain bioactive PSC (Sivakumaran et al., 2004). *Calliandra calothyrsus* was identified asthemost antimethanogenic amongst tropical forages (Durmic et al. 2012). It contains condensed tannins and has defaunating properties (Monforte-Briceño et al., 2005).

Experiment BA 15 - Five plants and PEG

Aim: to examine if the antimethanogenic effect diminishes in five plants after the addition of a tannin binder PEG

Results and Discussion

Production of methane was significantly higher with PEG than without it in 4 plants, while in *C. intybus* there was a significant reduction with PEG addition (Figure 5.1).

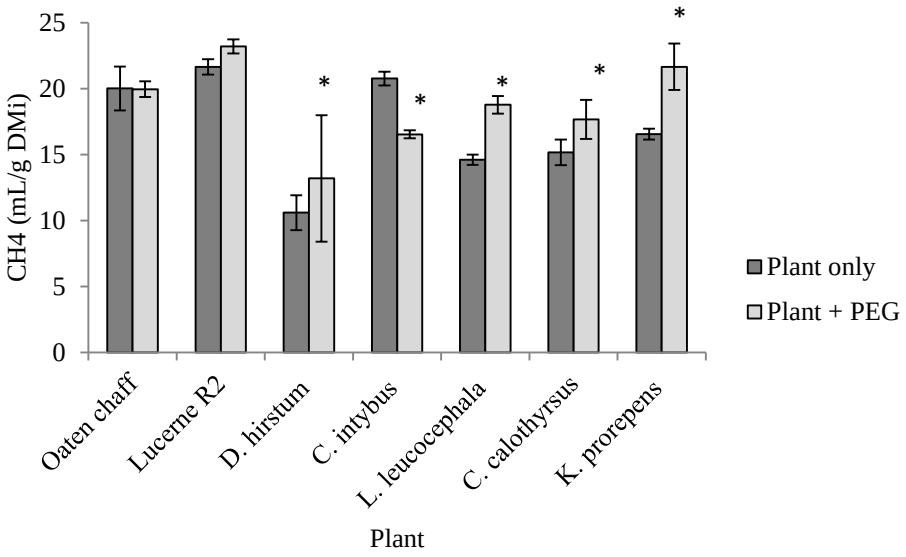

Figure 5.1. Methane production (mean ± SEM) by rumen microbes when fermenting different plants alone or in presence of PEG *in vitro.* * - 'Plant + PEG' treatment significantly different to respective 'Plant only' treatment

Gas production was increased after addition of PEG in *C. calothyrsus, D. hirstum,* and *K. prorepens* only (Table 5.1).

Table 5.1. Gas production by rumen microbes when fermenting different plants alone or in the presence of PEG *in vitro.* * - 'Plant + PEG' treatment significantly different to respective 'Plant only' treatment

Treatment	Gas (mL/g DM)		
	Plant only	Plant + PEG	
Oaten chaff	322	332	
Lucerne R2	323	326	
C. calothyrsus	274	300	*
C. intybus	333	332	
D. hirstum	291	309	*
K. prorepens	299	323	*
L. leucocephala	304	309	

In the current experiment, we have confirmed that tannins are likely to be responsible for the antimethanogenic effect in at least four plants tested here, with the greatest difference after PEG addition on methane observed with *K. prorepens* and *L. leucocephala*, where one third of the antimethanogenic activity was lost after addition of PEG. However, even after the addition of PEG, not all antimethanogenic activity was lost, as all four plants remained significantly lower compared to lucerne. Amongst the four plants, removal of tannins also removed some of the negative effects on microbial activity (gas production), except in *L. leucocephala*. *C. intybus* did not lose activity after adding PEG and it is not clear why it was in fact enhanced with the addition of PEG. Interestingly, PEG had an effect on lucerne as well, which produced slightly more methane after adding PEG. This is significant as lucerne is often used as a control fodder in methane trials, but this may indicate the presence of small amounts of tannins even in this legume, and should be taken into consideration in future trials.

In conclusion, tannins appear to be responsible for a major part of the antimethanogenic effects in four plants, however not all activity seems to be due to tannins. The inhibitory effect on microbial gas in these plants, especially in *L. leucocephala* also does not appear to be linked solely to tannins.

Experiment BA 20 - Crude extracts of four plants

Aim: to examine further the type of compounds that may be involved in antimethanogenic properties in these plants.

Materials and methods

We selected three plants with potential tannin-based bioacivity, and one plant where tannins might not be involved (Table 5.1). Plants were extracted using different solvents and tested as described previously.

Results and Discussion

Treatments varied in their gas production and methane output. None of the extracts from *C. intybus* were active, but MeOH/CHCL3 (1:1) extractof *D. hirstum* (A2), Acetone/aq (7:3) extract of *K. prorepens* (A12), and both acetone/aq (7:3) (A9) and MeOH/CHCL3 (1:1) (A8) extracts of *L. leucocephala* were active. All extracts that reduced methane also reduced overall gas production, but in all active extracts, the reduction in gas was less than 10% when compared to the control, except *K. prorepens* (reduction in gas of 20%).

Table 5.1. Microbial gas and methane (mean±SEM) produced from *in vitro* fermentation of oaten chaff in presence of different crude extracts from 4 plants. * - mean significantly different to control (P<0.05). DMi - dry matter incubated. Control - oaten chaff + EtOH

			Gas			CH_4		
Label	Plant species	Solvent	(kPa)		SEM	(mL/g DMi)		SEM
PC	Control	-	96		1.3	47.8		0.5
A 6	*Cichorium intybus*	Acetone/aq (7:3)	100		0.6	45.8		0.1
A 4	*Cichorium intybus*	MeOH/aq (8:2)	97		2.8	45.6		0.1
A 5	*Cichorium intybus*	MeOH/CHCL3 (1:1)	95		2.6	45.9		0.6
A 3	*Dorycnium hirstum*	Acetone/aq (7:3)	93		1.9	46.3		0.6
A 1	*Dorycnium hirstum*	MeOH/aq (8:2)	92		2.7	59.3		0.6
A 2	*Dorycnium hirstum*	MeOH/CHCL3 (1:1)	87	*	0.9	10.8	*	5.6
A 12	*Kennedia prorepens*	Acetone/aq (7:3)	76	*	0.7	37.0	*	0.2
A 10	*Kennedia prorepens*	MeOH/aq (8:2)	100		1.6	62.0		1.0
A 11	*Kennedia prorepens*	MeOH/CHCL3 (1:1)	96		0.6	50.2		0.1
A 9	*Leucaena leucocephala*	Acetone/aq (7:3)	90	*	1.4	2.6	*	0.8
A 7	*Leucaena leucocephala*	MeOH/aq (8:2)	97		1.7	47.5		1.2
A 8	*Leucaena leucocephala*	MeOH/CHCL3 (1:1)	90	*	0.8	6.6	*	1.9

This experiment outlined some potent candidates - *L. leucocephala* and *D. hirstum* that should be investigated further. Both produced extracts that reduced methane up to 90%. While the most potent extract in *D. hirstum* was MeOH/CHCL3 (1:1), similar to trends that we have observed with *E. glabra* and *B. pelecinus*, the most active extract of *L. leucocephala* was in fact Acetone/aq (7:3). This may imply a different active constituent is acting against methane production in this plant.

Experiment BA 21/22 - First fractionation of *L. leucocephala* and*D. hirstum*

Aim: obtain and test in IVFT the first set of fractions from *L. leucocephala* and *D. hirstum*

Results and Discussion

A total of 8 fractions from *L. leucocephala* and 7 fractions from *D. hirstum* were obtained (Table 5.2). Only two fractions, namely fraction 6.1/2 Dor-sep-MeOH from *D. hirstum* and 5/2 L-aq from *L. leucocephala* showed some small, but significant reduction in methane. There were 5 fractions that also caused a small but significant reduction in gas.

Table 5.2. Microbial gas and methane (mean±SEM) produced from *in vitro* fermentation of oaten chaff in presence of different crude extracts from 2 plants. * - significantly lower than the control (P<0.05). DMi - dry matter incubated. Control - oaten chaff + EtOH

Fraction	Code	Gas (kPa)		SEM	CH_4 (mL/g DM)	SEM	
Control		100		1.7	53	0.4	
10/2	Dor-aq	97		1.0	53	0.3	
9/2	Dor-ETOAC	95	*	0.4	52	1.0	
8/2	Dor-hexane	98		0.8	54	0.6	
7.1/2	Dor-sep-aceton	94	*	3.1	52	1.1	
7.2/2	Dor-sep-aceton	98		0.1	54	0.2	
6.1/2	Dor-sep-MeOH	96		2.1	49	2.1	*
6.2/2	Dor-sep-MeOH	98		1.5	53	0.4	
5/2	L-aq	93	*	2.1	41	2.2	*
4/2	L-ETOAC	95	*	0.7	50	0.9	
3/2	L-hexane	97		1.1	53	0.2	
2.1/2	L-sep-aceton	99		1.1	56	2.3	
2.2/2	L-sep-aceton	96		1.2	51	1.9	
2.3/2	L-sep-aceton	100		1.9	53	0.8	
1.1/2	L-sep-MeOH	94	*	1.2	56	1.2	
1.2/2	L-sep-MeOH	97		0.6	55	0.3	

The fractions from two plants showed only limited effects on methane. In *L. leucocephala*, the reduction was around 23%. This is not surprising, given that the *L. leucocephala* plant produced a similar level of inhibition when compared to lucerne (Experiment BA 15). However, inhibition with *D. hirstum* fraction was only 10%, while the whole plant inhibitedmethane by nearly 50% compared to lucerne (BA 15) and MeOH/CHCL3 (1:1) extract in BA 21 caused 80% reduction. Further selection of solvent and conditions is required to obtain a more potent extract from this plant.

Experiment BA 23 and 24b: *L. leucocephala* and *D. hirstum* fractions from ETOAC and CHCl3/MeOH

Background

In our previous experiment (BA 22), *L. leucocephala* was subjected to extraction by the active solvent extraction acetone/water (7:3+.01% TFA). The crude extract was dissolved in water and then extracted by hexane followed by EtOAC (liquid-liquid extraction technique) to remove the tannins compounds. In that experiment, there was some activity in *L. leucocephala* aqueous layer (L-aq).

Therefore, the experiment is repeated, but this time the crude extract was obtained by the most bioactive solvent extraction.

Materials and methods

Plant material (1.053g) of leucaena was extracted by 10 ml of acetone/water (7:3+(0.05-0. 1%) TFA (trifluoroacetic acid) and then left over night. The extract was filtered and then evaporated to remove the acid. The crude extract was dissolved in water (3 mL) and the extracted with hexane (3 X 5 mL). After that, the aq layer was partitioning to ETOAC (3 X 7 mL). The tannins were left in the aq layer with some high polar compounds. Fractions obtained are listed in Table 5.3. Plant material (5.095 g) of *D. hirstum* was extracted with 150 mL of $CHCl_3$/MeOH (1:1). The crude extract was dissolved in 5 mL dichloromethane and then was added to column contain silica gel 4.4.

Fractions were tested separately in the IVFT, after being dissolved and this solution included at 100 µL/100 mg substrate. In the assay, a positive control (substrate + ethanol) and an antimethanogenic compounds control (AMC, 50 µL teatree oil/100 mg substrate) were included.

Table 5.3. Fractions collected from leucaena for BA 23

Number	Sample code	Sample type	Weight (mg)
1/3	L. TV oo12	Leucaena crude extract was obtained by acetone/water (7:3+ (0.05-0. 1%)TFA)	14.9
2/3	L-hexane	The hexane layer	2.4
3/3	L-ETOAC	ETOAC layer	3.2
4/3	L-Aq	Aq layer	13.0

Table 5.4. Fractions from *D. hirstum* used in BA 24b

Sample code	Solvent	Weight
DH1	100% Petrol	2.6 mg
DH2	80% Petrol/20% ETOAC	0.7 mg
DH3	40% Petrol/60% ETOAC	3.5 mg
DH4	40% Petrol/60% ETOAC	13.8 mg
DH5	60% Petrol/40% ETOAC	0.4 mg
DH6	80% Petrol/20% ETOAC	-
DH7	100% ETOAC	0.3 mg
DH8	10% ETOAC/90% MeOH	8.1 mg
DH9	20% ETOAC/80% MeOH	5.6 mg
DH10	50% ETOAC/50% MeOH	10.4 mg
DH11	100% MeOH	10.9 mg

Results and Discussion

A total of four fractions from *L. leucocephala* and 11 from *D. hirstum* were tested, but none of the fractions caused significant reduction in methane (Figure 5.2).

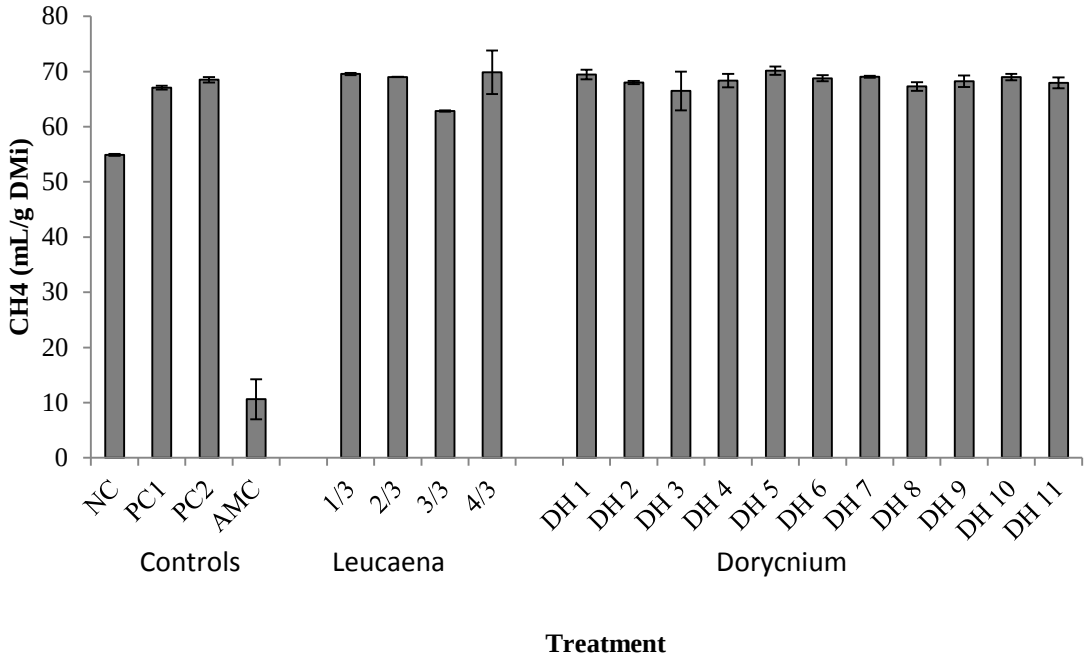

Figure 5.2. Methane production (mean ± SEM) by rumen microbes when fermenting oaten chaff in presence of different fractions from leucaena or *D. hirstum*.Controls - oaten chaff + EtOH. DMi- dry matter incubated

A different approach to extraction is required and/or fractions should be included at higher concentrations

Experiment BA26 - *L. leucocephala* and *D. hirstum* fractions at higher concentrations

Aim: to test *L. leucocephala* and *D. hirstum* fractions at higher concentrations.

Materials and Methods

Around 5 g of *L. leucocephala* was extracted with (150 mL) 50% $CHCl_3$/MeOH. The crude extract was loaded to the column by absorption method. A series of elution system (as listed in the Table 5.5) was used in collecting the fractions and up to 40% of each fraction was tested for bioactivity. Leucaena acetone/ aq extract was generated again using the same procedure as detailed in BA 21. Fractions L-hexane (2/3), L-EtOAc (3/3) and L-aq (4/3) were concentrated, where possible. In addition, fraction L-aq (5/2) from experiment BA 21 was re-tested at higher a concentration (total amount listed in the table dissolved in 500 µL EtOH, then added at 100 µL/100 mg substrate) to determine the effect concentration has on methane production. An antimethanogenic compound control (tea tree oil; 50µL/100 mg substrate) was included in the assay.

Table 5.5. Fractions obtained from *L. leucocephala* for testing in BA 26

Code	Sample	Description	Elution solvent	The tested concentration (mg)
Silica gel fractions				
LS 1	L. leucocephala 1	1st	Petrol	5.9
LS 2	L. leucocephala 2	2nd	80% Petrol/ETOAC	7.7
LS 3	L. leucocephala 3	3rd	60% Petrol/ ETOAC	27.5
LS 4	L. leucocephala 4	4th	40% Petrol/ ETOAC	3
LS 5	L. leucocephala 5	5th	20% Petrol/ ETOAC	1.7
LS 6	L. leucocephala 6	6th	ETOAC	1.9
LS 7	L. leucocephala 7	7th	90% ETOAC/ MeOH	12.7
LS 8	L. leucocephala 8	8th	70% ETOAC/ MeOH	64.5
LS 9	L. leucocephala 9	9th	50% ETOAC/ MeOH	41.8
LS 10	L. leucocephala 10	10th	20% ETOAC/ MeOH	16.5
LS 11	L. leucocephala 11	11th	MeOH	15.9
Crude extracts				
LL 1[A]	L. leucocephala 12	Leucaena crude extract	Acetone/aq 7:3 +o.1 TFA	169
LL 2[B]	L. leucocephala 13	L-hexane (2/3)	Hexane	2.4
LL 3 [C]	L.L 14	L-EtOAc (3/3)	EtOAc	4.6
LL 4 [C]	L.L 15	L-aq (4/3)	aq	49.1

[A] - Prepared fresh (new sample)
[B] - The same sample and concentration used in BA 23 experiment
[C]- The same sample used in BA 23 experiment, but more concentrated

D. hirstum crude extract was fractionated by silica gel column. The concentration of the previous tested fractions was 10% of the original (Table 5.6). However, this time up to 40% of the fractions was tested. Also, fractions having the same HPLC profiles were combined.

Table 5.6. Fractions obtained from *D. hirstum* for testing in BA 26

Code	Solvent	Weight	Tested in BA 23	Amount tested in BA 26
DH1	100% Petrol	2.6 mg	0.26	1.04
DH2	80% Petrol/20% ETOAC	0.7 mg	0.07	0.28
DH3	40% Petrol/60% ETOAC	3.5 mg	0.35	1.4
DH4	40% Petrol/60% ETOAC	13.8 mg	1.38	5.52
DH5	60% Petrol/40% ETOAC	0.4 mg	0.04	0.16
DH6	80% Petrol/20% ETOAC	-	na	na
DH7	100% ETOAC	0.3 mg	0.03	0.12
DH8	10% ETOAC/90% MeOH	8.1 mg	0.81	3.24
DH9	20% ETOAC/80% MeOH	5.6 mg	0.56	2.24
DH10	50% ETOAC/50% MeOH	10.4 mg	1.04	4.16
DH11	100% MeOH	10.9 mg	1.09	4.36

Results and Discussion

A total of 24 fractions from leucaena and *D. hirstum* were obtained, however none of these had inhibitory effect on methane production (Figure 5.3). Despite doubling the concentrations, there was no effect.

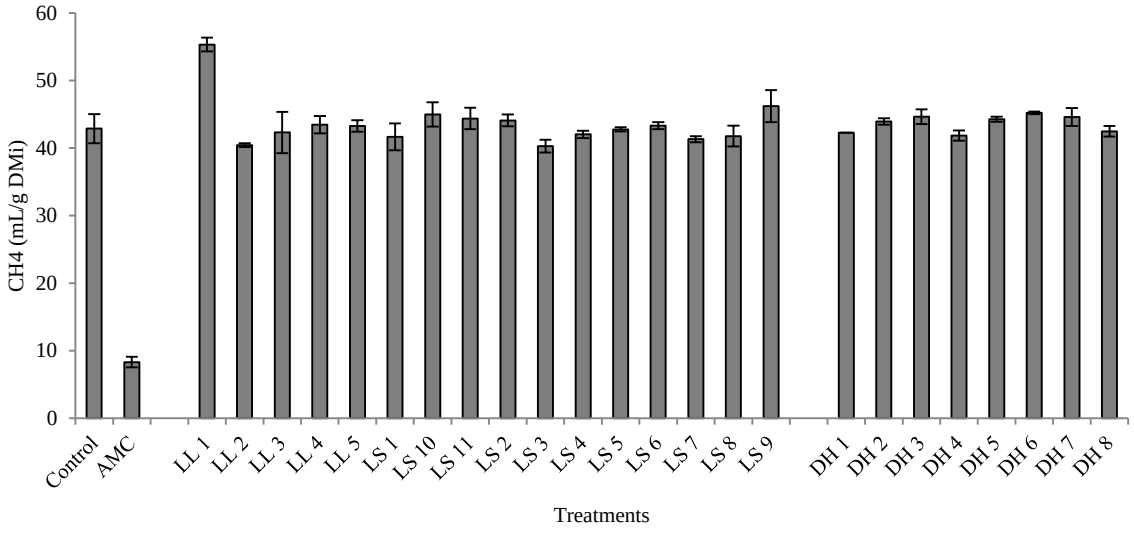

Figure 5.3. Methane production (mean ± SEM) by rumen microbes when fermenting oaten chaff in presence of different fractions from leucaena or *D. hirstum*.Controls - oaten chaff + EtOH. DMi- dry matter incubated

Experiment BA 28 - *In vitro* testing of *L. leucocephala* and *D. hirstum* - crude extracts retesting

Aim: to confirm the activity of original crude extracts, since there was no activity in any of the follow-up fractions (BA 25 and 26).

Results and Discussion

There was a significant reduction with the crude extract of *D. hirstum*, but not with any of the *L. leucocephala* ones (Table 5.7).

Table 5.7. Microbial gas and methane (mean±SEM) produced from *in vitro* fermentation of oaten chaff in presence of different crude extracts from 2 plants. * - significantly lower than the control (P<0.05). DMi - dry matter incubated. Control - oaten chaff + EtOH

Treatment	Fraction	Gas (mL/g DMi)		CH_4 (mL/g DMi)	
Control		389		50	
Leucaena					
Crude extract	Le - 01	378		46	
EtOAc layer	Le - 02	381		48	
Aq layer	Le - 03	382		48	
Dorycnium					
Crude extract	DoH - 01	377		9	*
EtOAc layer	DoH - 02	375	*	46	
Aq layer	DoH - 03	384		48	

The activity of crude extract of *D. hirstum* was confirmed, but the activity was not in the organic or aqueous layer. Further optimisation of extraction is required

Experiment BA 29 - *L. leucocephala* and *D. hirstum* using two types of columns

Background

Some of the pervious experiments showed that leucaena crude extract,MeOH/ $CHCl_3$ (1:1) and acetone/aq/TFA (7:3:0.05), did not show activity, but it could have been associated with the way the fractions were prepared. In this experiment the effect of stirring of these (acetone/aq/TFA (7:3:0.05)), was tested. In the previous experiment BA26 we confirmed the activity of *D. hirsutum* crude extract, but also showed the activity was lost during the isolation method. Therefore, in this experiment the MeOH/$CHCl_3$ (1:1) crude extract was fractionated by two different types of column chromatography: silica gel and polyamide. Polyamide column chromatography is more specific for phenols (tannins).

Aim: to compare the two isolation methods and to find out the best for isolating the active compounds.

Materials and Methods

L. leucocephala - Plant material was extracted with acetone/aq/TFA (7:3:0.05), under three different regimes - stirring, no stirring and stirring under nitrogen (Table 5.8).

Table 5.8. *L. leucocephala* extracts tested in the current study

Code	Treatment
Leo-1.3-1	Stirring with nitrogen
Leo-1.3-2	No stirring
Leo-1.3-3	Stirring without nitrogen

D. hirstum

Silica and polyamide fractions were obtained and tested (Table 5.9).

Table 5.9. Silica gel fractions from *D. hirstum* used in the assay. * note - some silica comes with last fraction

Code	Elution solvent	Sample mass (mg)
Silica gel fractions		
4DH-21S	Petrol	0.9
4DH-22S	30% EtOAC/ Petrol	10.1
4DH-23S	40% EtOAC/ Petrol	13.2
4DH-24S	EtOAC	1
4DH-25S	10% / MeOH /EtOAC	0.9
4DH-26S	MeOH	61.3
4DH-27S	10% / MeOH /EtOAC	115.2*
Initial crude extract		195 mg
Recovery		103.9%*
Polyamide fractions		
6DH-P1	water	49.1
6DH-P2	10% MeOH/ aq	15.7
6DH-P3	20% MeOH/ aq	

Code	Elution solvent	Sample mass (mg)
6DH-P4	50% MeOH/ aq	8.1
6DH-P5	70% MeOH/ aq	
6DH-P6	MeOH	
6DH-P7	70% MeOH/ $CHCl_3$	3
6DH-P8	50% MeOH/ $CHCl_3$	5
6DH-P9	$CHCl_3$	7.2

Results and Discussion

There was some small reduction with *L. leucocephala* extract when stirring was excluded (Table 5.10). In *D. hirstum*, fraction 6 DH - P9 appeared very potent.

Table 5.10. Microbial gas and methane (mean±SEM) produced from *in vitro* fermentation of oaten chaff in presence of different crude extracts from 2 plants. * - significantly lower than the control (P<0.05). DMi - dry matter incubated. Control - oaten chaff + EtOH

Treatment	Gas (mL/g Dmi)	CH_4 (mL/g Dmi)	
Control	383	52	
Leucaena extracts			
Le - 01-3-1	398	52	
Le - 01-3-2	398	49	*
Le - 01-3-3	410	52	
Dorycnium fractions			
4DH-21S	386	53	
4DH-22S	387	52	
4DH-23S	387	51	
4DH-24S	383	53	
4DH-25S	388	53	
4DH-26S	412	65	

Treatment	Gas (mL/g Dmi)	CH_4 (mL/g Dmi)	
4DH-27S	425	71	
6 DH - P1	399	57	
6 DH - P2	390	53	
6 DH - P3	390	54	
6 DH - P4	383	51	
6 DH - P5	394	54	
6 DH - P6	387	53	
6 DH - P7	389	53	
6 DH - P8	391	53	
6 DH - P9	376	7	*

Discussion

In the current experiment, the results were limited to one fraction only, 6 DH - P9. This is a fraction obtained using polyamide gel, which is more specific for tannin. This may align with reports that the bioactive compound in dorycnium (*D. rectum*) is tannin (Sivakumaran et al., 2004).

General discussion

The studies conducted in this part of the project indicated that tannins are likely to be responsible for the antimethanogenic effect in at least four plants tested here, with the greatest difference after PEG addition on methane observed with *K. prorepens* and *L. leucocephala*, where one third of antimethanogenic activity was lost after addition of PEG. However, even after the addition of PEG, not all antimethanogenic activity was lost, as all four plants remained significantly lower to lucerne. In *C. intubus*, the activity was not affected with the addition of PEG, which supports the reports that tannins are negligible in this forage (Waghorn et al., 2002).

While subsequent fractionation revealed some potent fraction candidates initially in *L. leucocephala* and *D. hirstum*, further fractionation and testing failed to progress any of these, except for one fraction in *D. hirstum* (6 DH - P9). This is a fraction obtained using polyamide gel that is more specific for tannin, which aligns with reports that bioactive compound in dorycnium (*D. rectum*) is a type of tannin (Sivakumaran et al., 2004). It appears that a different approach and methodology may be required to isolate and maintain activity of fractions in these plants. In addition, there are many examples in the literature of variation in antimethanogenic effect in these plants. For example, while *C. intybus* has been implied as a potential methane-mitigating forage (Waghorn et al., 2002), in some more recent reports it failed to produce a bioactive effect when fed to the sheep (Sun et al., 2011). In a similar manner, while tannins from *L. leucocephala* are found to be inhibitory on methane-producing or methane enhancing microbial populations *in vitro* (Tan et al., 2011), a subsequent *in vivo* study failed to confirm their role in methane mitigation (Soltan et al., 2013). One possible explanation may lay in differences between plant cultivars used or seasons when plants were collected (Gebrehiwot et al., 2002) as well as methodologies applied. As forage tannins are a diverse group of compounds (Reed,

1995), it may be difficult to have a 'blanket-type' approach for isolating these and we may need to develop a more specific approach and assays for each plant type.

Nevertheless, we have confirmed that the activity in some of these plants is mainly linked to tannins, similar to what has been reported in the literature. Further studies should focus on examining variability between different cultivars and develop more specific methodologies to target antimethanogenic compounds in these plants.

Implications

Tannins are likely to be responsible for antimethanogenic effect in *L. leucocephala*, *D. hirstum*, and *Kennedia prorepens*. One fraction with a high antimethanogenic effect was isolated from *D. hirstum*. The methodology needs to be further optimized/species-tailored to be able to further access bioactive components in these plants.

6. *In vitro* testing of grape marc

Experiment BA 27a - *In vitro* testing of commercial wine tannin Graptan-S

Background

Grape marc is considered as valuable feed supplement in cattle (Hentges et al., 1982) and sheep (Ryan et al., 2004). In particular, it has low rumen degradability of crude protein because of relatively high tannin concentrations. Other favourable effects have been reported. In our previous studies, we have observed that *in vitro* incubation of grape marc results in 30% less methane when compared to fermentation of oaten chaff (RELRP Final report). However, due to high water content and quick spoilage, the use of fresh grape marc may be limited at farms close to wineries. Further, Pellikaan et al.(2011) reported that grape seed tannins reduce methane from the *in vitro* incubations with mixed rumen population. A current NLMP project is investigating chemical profiles of grape marc in Australia and using grape marc tannins *in vitro* as model for examining the tannin effect on rumen methane.

The next step would involve testing grape marc tannins *in vivo*. In discussion with AWI it was concluded that it may not be possible to scale up extraction and production of grape marc tannins in reasonable time. We discussed an approach whereby we would analyse commercially available wine tannins in the *in vitro* fermentation batch culture (IVFT) and if they reduce methane, the chemical profile would be analysed to compare with grape marc currently being investigated in NLMP.

The aim of the current study was to examine the effect of commercial grape skin tannins on *in vitro* fermentation in rumen batch culture.

Materials and Methods

A commercially available tannin from grape skin (Grap'tan® S, FERCO Œnologie, Quartier Viressac, Saint Montan, France), containing >65% tannin (Do 280 – Eq. catechins) was obtained and tested in the IVFT (Durmic et al., 2013). The tannin was added to a high-protein concentrate-based diet (EasyOne, Milne Feeds, Welshpool, Australia; 14.5% CP; ME 11.0 MJ/kg; crude fibre 20.0%). The tannin was included at levels of 100 mg and 200 mg per g of substrate (10 mg or 20 mg per 10 mL buffered rumen fluid). These amounts correspond to the amounts found to be effective in the *in vitro* studies using grape seed tannins (Pellikaan et al., 2011), or acacia tannin *in vitro* (Durmic et al., 2013) and *in vivo*(Grainger et al., 2009).

Results

There was some small reduction (16%) in methane when concentrate diet was supplemented with Grap'tan® S at 200 mg per g food. This was accompanied with a significant reduction in gas (Table 6.1).

Table 6.1. Gas and methane production from a concentrate diet supplemented with Grap'tan® S at various concentrations. *-significantly lower than the control (P<0.05)

	Gas (mL/g Dmi)	CH_4 (mL/g Dmi)
Control	418	65
100 mg/g	407	61
200 mg/g	390 *	54 *

Discussion

Inclusion of a commercially available, 'off-the-shelf' wine tannin derived from grape skin, with a concentrate diet, and at a relatively high level (20%) caused only a small reduction in methane. Further investigations around other commercial grape tannins may find other grape products that are more potent.

Implications

Some reduction was observed with Grap'tan® S, but the search for commercial grape tannins should expand with the possibility of finding a more active one.

Experiment BA 30a*In vitro* testing of 3 commercial grape tannins: Graptan-S, Graptan-E and Graptan-PC

Background

In our preliminary experiment (BA 27a), we examined a commercially available grape tannin derived from white grape skin (Grap'tan® S, FERCO Œnologie, Quartier Viressac, Saint Montan, France), containing >65% tannin (Do 280 – Eq. catechins, http://www.enoltech.com.au/images/stories/GRAPTAN%20S%20eng.pdf). We tested the product added to a high-protein concentrate diet as a substrate, at levels of 100 and 200 mg/g, using IVFT. We observed approximately 17% reduction in CH_4 production with addition of this product at a level of 200 mg/g.

The aim of the current study was to examine the effect of other commercial grape tannins on *in vitro* fermentation in batch culture and expand the screening to include a higher fibre-based substrate. The objectives of this study were to help establish that grape tannins are responsible for the antimethanogenic effect and assist in moving towards an *in vivo* application by using commercial analogues.

Materials and Methods

Three commercially-available wine tannins obtained from FERCO Œnologie, Quartier Viressac, Saint Montan, France, i.e. one derived from grape skin of white grape (Grap'tan® S) and two derived from grape seeds (Grap'tan® E and Grap'tan® PC) were tested using IVFT (Durmic et al., 2013). They were added to two different substrates; a fibre-based (oaten chaff) and a concentrate-based (EasyOne, Milne Feeds, Welshpool, Australia; 14.5% CP; ME 11.0 MJ/kg; crude fibre 20.0%). The wine tannins were included at a level of 200 mg/g of substrate (20 mg per 10 mL buffered rumen fluid). These amounts correspond to the amounts that were effective in the *in vitro* studies using grape seed tannins (Pellikaan et al., 2011), or acacia tannin *in vitro* (Durmic et al., 2013) and *in vivo*(Grainger et al., 2009) and that was confirmed in our previous study. The aliquots of each sample were also sent to AWRI for chemical analyses.

Results

Addition of any of the three grape tannins to any of the two control substrates resulted in a significant reduction in gas and CH_4, but the reduction in CH_4 was always greater than the reduction in gas (Table 6.2). The most potent was Grap'tan - PC, producing only 19 mL (added to fibrous diet) or 29 mL (added to concentrate diet) of CH_4 per g of DM incubated (DMi) compared to controls that produced 42 mL and 50 mL, respectively. This resulted in a 55% (with concentrate substrate) and 41% (with oaten chaff substrate) reduction in CH_4 production (or 36% and 31% reduction in CH_4 concentration in headspace gas). The other grape seed tannin, Grap'tan E was also more effective than the grape skin source (Grap'tan-S). The effect on methane production in all treatments was accompanied with a less than 20% reduction in total gas production (except in Grap'tan-PC x fibrous substrate). There were no obvious differences in efficacy of tannins between the two different types of substrate.

Table 6.2. Gas and CH_4 from *in vitro* fermentation of oaten chaff and concentrate substrate fermented in presence of different grape tannins. * - within the row, significantly lower than the control ($P<0.05$)

Fermentation parameter	Treatment						
	Control	Grap'tan - S		Grap'tan - E		Grap'tan - PC	
Fibrous substrate							
Gas production (mL/g DMi)	375	328	*	316	*	287	*
CH_4 production (mL/g DMi)	42	32	*	28	*	19	*
CH_4 concentration (mL/100 mL gas)	11	10	*	9	*	7	*
Gas inhibition (%)	-	13		16		23	
CH_4 production inhibition (%)	-	24		34		55	
CH_4 concentration inhibition (%)	-	12		18		36	
Concentrate substrate							
Gas production (mL/g DMi)	389	368	*	351	*	325	*
CH_4 (mL/g DMi)	50	41	*	34	*	29	*
CH_4 concentration (mL/100 mL gas)	13	11	*	10	*	9	*
Gas inhibition (%)	-	5		10		16	
CH_4 production inhibition (%)	-	17		33		41	
CH_4 concentration inhibition (%)	-	15		23		31	

There was a clear relationship between the amount of tannin and their activity in IVFT (Figure 6.1; J. Hixson, pers. comm.).

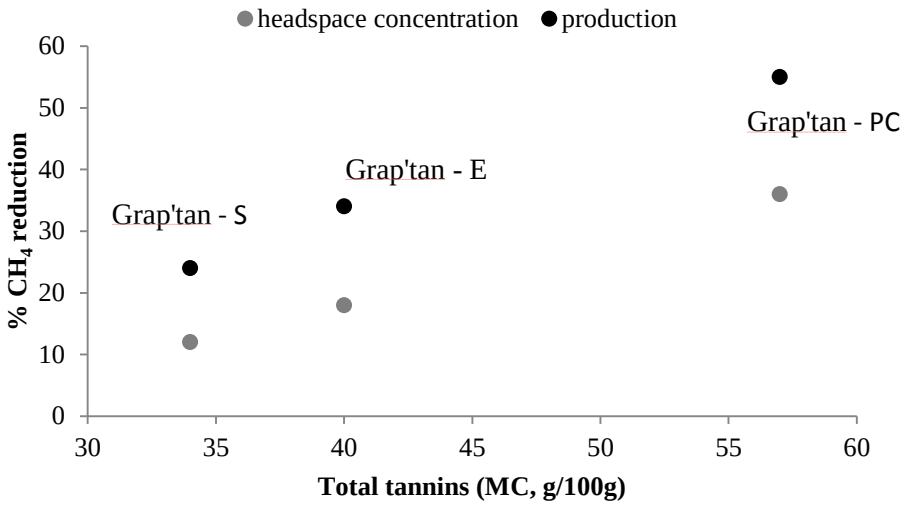

Figure 6.1. Relationship between level of tannin in commercial wine tannin products and methane reduction (%) when fermented *in vitro* with a fibrous diet

Discussion

We have demonstrated that some commercial tannins from grape skin or seed can affect CH_4 production *in vitro*. We have confirmed the findings from our preliminary testing with a tannin product derived from grape skin, Graptan-S (17% reduction in CH_4 in both studies), but here we identified two other potentially more potent commercial tannins that are derived from grape seed. Graptan-PC was the most potent, causing 40-50% reduction in CH_4 production (31-36% reduction in CH_4 concentrations) and was more effective than the other grape seed derived product, i.e Graptan-E. It would be interesting to do a more detailed chemical analysis of these three tannins to find out what differences there are in their composition, because this information may lead to the identification of the types of tannin compound(s) that may be responsible for antimethanogenic bioactivity. While the level of inclusion tested here was still relatively high in practical terms, it helped to identify the candidates for further analysis and testing. It is possible that the more potent candidates would be effective at lower doses and/or their active compounds may be further isolated and used. This aligns with the findings of the AWRI group that chemical composition of grape seed tannins differ to that of grape skin (i.e. high in gall tannin component), and the findings of University of Melbourne that the gall tannin component was strongly negatively related to CH_4 production when added with a concentrate-based diet in an *in vitro* fermentation system (project 01200.007; B.CCH.6410).

It should be noted that the antimethanogenic effect was accompanied with some reduction in overall gas, which could be partly attributed to the reduction in CH_4. However, as CH_4 was not completely reduced in any of the treatments, and the reduction in gas was greater in some treatments than the CH_4 portion in the control, it appears that there is some general inhibitory effect of the products on rumen microbial fermentation. In this study, we only measured gas as an indicator of microbial activity; a complete suite of parameters such as dry matter digestibility, volatile fatty acid and ammonia concentrations should be examined to reveal the mechanism of action in more detail.

Under the experimental conditions we used, we did not observe any big differences in anti-methanogenic activity of additives when combined with the two different types of substrate. In general, it is believed that feed additives (in particular tannins) are more efficient when included in a concentrate-based diet, however this was true only for CH_4 concentration and not for overall CH_4

production, where the opposite occurred with two of the tannins tested here. This highlights the need to test additives over a range of diets/conditions, measuring a full suite of parameters over a period of time, before any claims and further advancement towards *in vivo* can be made.

Implications

Selected commercial wine tannins can reduce methane *in vitro* and can progress to larger scale *in vivo* testing as analogues of analytical quantities from the AWRI project.

11. Discussion

The objective of the current project was to isolate, describe and examine the effects of bioactive compounds responsible for antimethanogenic activity in selected plants. The results from our study confirmed that *E. glabra, B. pelecinus,* as well as*D. hirstum*, *C. intybus,L. leucocephala*, *Calliandra calothyrsus* and *K. prorepens*contain fractions with antimethanogenic activity. Selected fractions of *E. glabra and B. pelecinus* were capable of reducing methane production by rumen microbes by up to 90%, and some of these were also highly active against key species of rumen methanogens. While we have identified one purified compound from *E. glabra*, sesamin, we could not make any definite conclusion on its capacity to reduce methane from rumen microbes. A variety of other bioactive fractions from *E. glabra* were also isolated, offering a range of pathways for further investigation towards developing a potent methane inhibitor. The reduction in total gas and VFA (where tested) with all fractions that were antimethanogenic demonstrated that fractions of *E. glabra* may have an effect on rumen microbes, which is consistent with the results we have obtained when fermenting *E. glabra*whole plant material (Durmic et al., 2010) and activity towards bacterial populations in the rumen (Li et al. 2013), so care should be taken, and appropriate doses determined, that have less detrimental effect. It should be emphasised that the antimethanogenic activity in some fractions disappeared after multiple separation steps were undertaken and it seems that there is a combined effect among fractions of *E. glabra* that contribute to low methanogenesis. Some antimethanogenic *E. glabra* fractions affected microbial fermentation and require further investigation into finding an appropriate dose.

Conversely, the active fractions in *B. pelecinus* caused reduction in methane without affecting VFA production. *B. pelecinus* maintained this effect, despite alteration of its chemical compounds by microbes in the RUSITEC. Fractions from *B. pelecinus* were also potent inhibitors of key methanogens when tested in pure culture, and some of these fractions caused an effect that was close to lethal. The antimethanogenic bioactivity of biserrula fractions varied amongst different methanogens, and it is possible that the biserrula fractions are targeting only certain pathways in these. While were not able to isolate and identify any pure compounds from this plant, it should be noted that there was no single fraction from biserrula that targeted all methanogens, and a mix of compounds, once defined, may in fact have better prospects than a single compound. Overall, biserrula is obviously a very promising candidate in this respect and the work will continue beyond NLMP through a PhD student.

In this study, we assesed the antimethanogenic effect of fractions using several approaches - from mixed batch fermenation, RUSITEC, pure culture, by measuring methane, microbial cell growth and recovery of cells after exposure to fractions. While in general these aim to complement each other, sometimes they gave different results. Some of these differences may be explained by the variation in the methodologies and their limitations. While batch culture is a good screening tool and can provide a quick and easy tool for narrowing down the treatments to be tested further, it is focused on microbial activity and may not detect a reduction in microbial cell numbers. While it is assumed that reduced microbial cell growth will result in decreased overall activity, it appears that some variation may occur. It is also important to understand limitations of the molecular methods applied here. The

microbial cell growth was assesed based on numbers of gene copies, which does not take into account cell activity. Cells may still be viable, but slowed down or dormant. It would mean that even inactive cells may be counted, which may explain why we have observed a lack of response in methane with some treatments that apparently affected microbial cell growth. This is of particular interest when considering practical applications, as these dormant cells may in fact regain their activity after the bioactive compound is removed and be a subsequent cause of inefficiency of the method.

It is interesting that a fraction defined as 'inactive' in the IVFT (fraction 3) was actually active against three methanogens in pure culture, and other fractions that were highly active in the IVFT (fraction 2 and 8), did not affect all methanogens, in particular *M. gottschalkii*. While both testing systems we used are artificial, IVFT represents the microbial diversity and at numbers that are more representative of the numeric proportions that they would be in the rumen of the animal whereas when testing in pure culture microbial the cells are present as the same, constant, number across all species screened. As a consequence, microbes that are normally present in low numbers in the rumen would be articifically higher in pure culture and potentially not affected when present in larger numbers and with no competition. Rumen microbial profiling in sheep revealed a great diversity - for example, one study showed that sheep on pasture in Western Australia were dominated by members of the *M. gottschalkii*, while sheep in Queensland, had those related to *M. gallocaecium* and had only few members of the *M. gottschalkii* clade. This is of particular importance when developing a methodology, as it appears that there may not be a 'blanket' approach for all and that a mix of compounds (or even a whole plant) rather than a single compound may have better prospects in this respect. Further, the fraction assesed as active in pure culture but inactive in IVFT, may in fact be degraded by rumen microbes. Hence, in a pure culture, away from degradation, its activity is maintained, but it is inactive in a mixed rumen fermentation system. As a consequence, this fraction *in vivo* would also be ineffective. Overall, it appears important that testing is conducted at both levels to improve chances of finding and confirming the bioactivity, but is imperative to take these testings further and confirm them *in vivo*.

When developing a microbial-controlling additive, it is important to achieve persistent and cidal effects. Many attempts to do this in the past have failed for this reason, including the use of antibiotics, as they leave behind viable cells that eventually lead to resistance in microbes. In the current study, none of the fractions tested was lethal to the methanogens, and when the methanogens were removed from a broth containing active fractions, the cultures grewback and the methane-producing activity returned to normal. However, a concentration-dependent microbial growth was observed with other antimethanogenic agents such as bromochloromethane and chloroform (Denman et al., 2007; Knight et al., 2011), and it is reasonable to expect that with increased concentrations, cidal effects may occur with biserrula fractions.

An interesting finding in this study is that certain plant chemicals or metabolites may dissipate over time when exposed to fermentation by rumen microbes. It is possible that this is the evidence of a latent potency in microbes to adapt to particular plant compounds that are present in the system. This may help in understanding better particular microbial strategies embedded in rumen system and enable better predictions of potential pathways of adaptation *in vivo*.

We have demonstrated that some commercial products derived from plants can be potent antimethanogenic additives. These can be easily scaled-up for *in vivo* testing and progress towards developing a novel plant-based additive. As some of these or similar compounds are already in use as commercially available supplements (grape marc) or additives (i.e. CRINA Ruminants). The likelihood of their adoption is greatly enhanced. We did not observe any big differences in antimethanogenic activity of additives when combined with the two different types of substrate. In general, it is believed that feed additives (in particular tannins) are more effective when included in a concentrate-based diet, however this was not the case in our studies, as selected additives that were

tested in this manner were equally effective, regardless of the type of the substrate. In practical terms, this means that the same additive may be applicable for a range of different diets and suit variety of production systems.

In conclusion, we found that there is a great spectrum of plant compounds, effects and modes of action. Amongst these, it is possible to select those that can sustain in a rumen environment that can persistently reduce methanogen growth and activity, while supporting microbial fermentation in the rumen. Bioactive plant fractions/compounds may provide a viable, sustainable option for developing a sustainable CFI methodology that does not impede animal production.

12. Significance of findings for Australian agriculture

Our findings are significant for Australian agriculture because we have identified both plants and compounds isolated from essential oils that provide opportunities to reduce on-farm methane emissions in extensive grazing systems as well as more intensive ruminant production systems.

There appears to be more than one compound responsible for the antimethanogenic effects in the plant species *Biserrula*, *Eremophila* and *Leucaena*. This is encouraging as the methane mitigation effects maybe more persistent if there are multiple compounds involved because the rumen microbial population are likely to take longer to adapt. These plant species could be included in pasture and shrub-based systems now, and are likely to be most effective in combination with eachother and/or other pasture species (ie. as part of a mixture).

Perhaps the most exciting result was the identification of 3 single compounds from essential oils extracted from Australian native plants that have the potential to reduce methane production dramatically, particularly in more intensive livestock systems. The compounds ‘L’, ‘C’ and ‘G’ could all be incorporated in pelleted diets or added as a supplement to the diet of animals produced under intensive feeding systems. The advantage of identifying specific compounds, compared with including a whole plant species in a grazing system, is that we can add them at a known amount and expect a consistent reduction in methane. In contrast, the amounts of the compounds responsible for reducing methane in whole plants can fluctuate according to factors like the weather, season, stage of growth of the plant, soil type and access to water and nutrients. Essentially, we would have more control over the extent of methane mitigation if we can add specific amounts of a single bioactive compound. There are also possibilities for developing a new industry around the extraction of the bioactive compounds, if they are shown to have a significant impact on profitability.

13. Future research needs

The next obvious step would be testing some of the compounds and combinations *in vivo*, particularly C, L and G. Further experiments area also warranted that focus on determining appropriate source, dose, solvent type and other conditions, as well as rule out any toxic effect on rumen microbes in mixed and in pure cultures. While it still remains unclear which specific compounds were responsible for the antimethanogenic effect in the bioactive plants, further fractionation and testing in batch culture may help guide the purification of compounds from these plants. Alternatively, it may indicate that the effects of these plants come from more than one compound that when purified to single compounds lose their activity. However, identifying active fractions from these plants may also help us target other plants that contain similar compounds or compound mixes without the need to go through the extensive process we have had to undertake in this project.

Overall, the findings in the current study warrant further investigation of all of these fractions, with particular emphasis on essential oil compounds and fractions from biserrula. These investigations should also focus on examining variability between different cultivars and develop more specific methodologies to target antimethanogenic compounds in other plants.

14. Publications

Papers in peer-reviewed journals

Durmic, Z., Moate, P. J., Eckard, R., Revell, D. K., Williams, R., Vercoe, P. E., (2014) *In vitro* screening of selected feed additives, plant essential oils and plant extracts for rumen methane mitigation. J Sci Food Agric 94, 1191-1196.

Banik, B. K., Durmic, Z., Erskine, W., Ghamkhar, K., Revell, C., (2013) *In vitro* ruminal fermentation characteristics and methane production differ in selected key pasture species in australia. Crop Past Sci 64, 935-942.

Banik, B. K., Durmic, Z., Erskine, W., Nichols, P., Ghamkhar, K., Vercoe, P., (2013) Variability of *in vitro* ruminal fermentation and methanogenic potential in the pasture legume biserrula (*Biserrula pelecinus* L.). Crop Past Sci 64, 409-416.

Durmic, Z., Blache, D., (2012) Bioactive plants and plant products: Effects on animal function, health and welfare. Anim. Feed Sci. Technol. 176, 150-162.

Conference papers

Banik, B., Durmic, Z., Erskine, W., Revell, C., Vercoe, P., 2014. *Biserrula pelecinus* L. shows persistent low methane production under a continuous culture fermentation system (rusitec) when mixed with *Trifolium subterraneum* L., LIVESTOCK, CLIMATE CHANGE AND FOOD SECURITY CONFERENCE, 19-20 May 2014 Madrid, Spain, p. 19.

Durmic, Z., Jahani-Azizabadi, H., Osmani, A., Vercoe, P. E., 2012. *In vitro* screening of selected Australian essential oils as dietary additives for methane mitigation from ruminants, 8th INRA-Rowett Symposium on Gut Microbiology, Clermont-Ferrand, France, p. 113.

Durmic, Z., Vadhanabhuti, J., Lund, K., Humphries, A., Vercoe, P., 2014. Variability in methane production from ruminal fermentation of temperate forage legumes and grasses, LIVESTOCK, CLIMATE CHANGE AND FOOD SECURITY CONFERENCE, 19-20 May 2014 Madrid, Spain, p. 23.

Joy, M., Durmic, Z., Vadhanabuti, J., Vercoe, P., 2014. Associative effect of fermenting a low methanogenic plant biserrula pelecinus with selected forages in vitro, LIVESTOCK, CLIMATE CHANGE AND FOOD SECURITY CONFERENCE, 19-20 May 2014 Madrid, Spain, p. 32.

Presentations

Durmic, Z (2014) Novel approaches in methane mitigation from ruminants. CITA Zaragoza, Spain May 2014

Durmic, Z (2014) Using plants to reduce methane emissions from ruminants. UWA Plant Biology seminar series Nov 2014

Banik, B (2014) *Biserrula pelecinus* L., a pasture legume that can help reduce methane emission ('burps') from sheep. IOA Presentation PG Showcase 2014 May 2014

Banik, B (2014) *Biserrula pelecinus* L., an Australian pasture legume that reduces unwanted gas from sheep and cattle. UWA Animal Biology PG seminars Dec 2014

Durmic, Z (2013) Antimethanogenic plants and products for ruminants. AARN Meeting, Melbourne Mar 2013

Durmic, Z (2013) Methanogenic Archaea -Villains of the Third Kind. QEII Seminar Series Oct 2013

Durmic, Z (2013) Greenhouse gasses from sheep and cattle. Future Science Workshop UWA Nov 2013