

final report

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Impacts of Leucaena plantations on greenhouse gas emissions in northern Australian cattle production systems

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Executive summary

The project was conducted with steers grazing established Leucaena or grass pastures to determine the impact on herd scale methane emissions, productivity and rumen function.

Relative emissions from cattle with access to Leucaena appeared lower, particularly in the months following the wet season, compared with those grazing grass dominated pastures. Rumen microbial analysis showed Leucaena diets increased the relative abundance of methyl group-utilising Methanosphaera while decreasing the proportion of other functional groups of methanogens. In Leucaena fed cattle there was a shift in the bacterial populations and fermentation to more reduced end products which may contribute along with leucaena tannins to decreased methane emissions.

The effects of Leucaena-finishing cattle on emissions, production and profitability at the whole farm level was modelled using the Beef Greenhouse Accounting Framework and the resultant carbon offset income determined relative to baseline data assuming two C prices (\$/t CO₂-e). Finishing steers on Leucaena effectively increased animals carried and liveweight turnoff by 15% and 31%, respectively.

The addition of Leucaena to beef production systems has the potential to increase productivity and gross margin, whilst reducing emissions intensity. Provided net farm emissions are maintained or reduced, Leucaena appears conducive to sustainable intensification of beef production in tropical grazing systems.

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1. Background

In northern Australia, large areas of pastures based on *Leucaena leucocephala* (>200,000 ha) have been planted to support productive and profitable livestock systems producing "grass-fed" beef. The perennial nature of *Leucaena* provides an on farm source of feed throughout most of the year in tropical beef production systems. *Leucaena* pastures can produce up to 37,500 t of liveweight gain valued at >\$69 M each year. Since 2010 there has been an increase in area sown to *Leucaena* in Queensland. While the exact area under production at present is uncertain, current estimates put it at greater than 250,000 ha. There has also been significant interest and sowings in the Northern Territory and Western Australia, and some smaller areas in NSW (Reynolds 2013). The area planted across northern Australia, including Qld, WA and the Northern Territory is expected to expand by 300,000 to 500,000 ha over the next 10 years, particularly with the release of a psyllid-resistant variety of *Leucaena* (Reynolds 2013).

Regardless of the change in grazing management any benefits from production efficiencies can be associated with reductions in greenhouse gas (GHG) emissions, especially in terms of emissions intensity (GHG emissions per unit of production). Enteric methanogenesis represents an energy loss to the ruminant and can be affected by a number of factors, including level of feed intake, diet characteristics, addition of lipids or ionophores to the diet, changes in rumen micro flora and level of animal productivity (Johnson and Johnson, 1995; McAllister *et al.*, 1996).

Studies in open-circuit respiration chambers with *Bos indicus* steers have demonstrated that supplementation of a Rhodes grass with up to 44% *Leucaena* in the total diet can result in decreased methane emissions per unit digested organic matter intake (DOMI) by up to 15% compared with Rhodes grass only diets (Kennedy and Charmley 2012). These benefits would be enhanced through improved liveweight gain and reduced emissions per unit of animal product. In those studies methane yields (g/kg DOMI) for cattle were not significantly affected when Dolichos Lab lab, Burgundy bean or Stylo constituted increasing proportions of the diet. In contrast, methane yields from cattle consuming diets with *Leucaena* were lower than for other tropical legumes tested, and were negatively related to *Leucaena* inclusion rate (Kennedy and Charmley, 2012). The presence of lipid material in *Leucaena* forage (Mtenga and Laswai 1994) has been suggested as a factor contributing to reduced methane yields from this browse legume (Dong *et al.*, 1997), although the effect from condensed tannins and amino acids also require consideration.

To date there have been no direct longitudinal methane measurement campaigns to quantify GHG emissions from beef cattle grazing *Leucaena* dominated pastures typical of commercial scale operations. Open path lasers have been used successfully to measure methane emissions from feedlots (McGinn *et al.*, 2008), small numbers of cattle in relatively small paddocks (< 12 ha) over short periods of time (Tomkins *et al.*, 2011; McGinn *et al.*, 2011) and at the herd scale across regions typical of northern Australia (Charmley 2012).

Beef production systems could be evaluated in terms of yearly livestock productivity and herd methane emissions thereby demonstrating the effectiveness of farming practices involving *Leucaena* finishing on decreasing methane emission intensity and how this may contribute to abatement opportunities under an Emission Reduction Fund (ERF) framework. Emission reduction is a key component to methodology development for consideration under the Emission Reduction Fund and for individual farmers to claim Australian Carbon Credit Units (ACCU) for the offset in carbon emissions (Comm. Aust., 2014). The lack of relevant data from northern production systems will impact on the development of future mitigation and management strategies for *Leucaena* finishing systems.

The work provides an opportunity to monitor the microbial changes in the rumen that underpin the reductions in methane using different levels of *Leucaena* feeding. This information can be used to inform related research that aims to manipulate the rumen through improved digestive efficiency while reducing methane formation.

This project was conducted to deliver the following outputs as described in the Funding Deed;

1. Estimates of methane reduction by Leucaena feeding practice compared with equivalent grass dominated pastures.
2. Estimates of productivity benefits from Leucaena feeding practice.
3. Net GHG mitigation benefit of Leucaena feeding systems and management practices.
4. Identification of methanogens and the hydrogenotrophic bacteria in the digestive tract of ruminants which should be targeted in strategies to reduce methane emissions.

With this knowledge more informed models can be developed to better understand and manage changes within the rumen biome that result in methane abatement for beef production systems while improving farm productivity.

2. Methodology

i. Project sites

The project was conducted on two sites: Belmont Research Station, near Rockhampton QLD (LAT 23.2134° S; LONG 150.390258° E. elev 17 m); and Brian Pastures, near Gayndah QLD (LAT 25.6534° S; LONG 151.7457° E. elev 132 m), during 2013 and 2014. Both sites had established pastures: irrigated Leucaena (*L. leucocephala* cv Cunningham) and Rhodes grass (*Chloris gayana*) pastures; or dryland Leucaena (*L. leucocephala* cv Cunningham and Taramba) and native grasses (*Dicanthium* sp.), respectively.

Experimental paddocks used on Belmont were 7 ha containing double rows of Leucaena at 4m spacing with inter rows containing Rhodes and Green panic (*P. maximum*), or paddocks of approximately 6.85 ha dominated by Rhodes grass. Prior to commencement of the project (2010) Rhodes grass pastures were fertilised with CK88 (100 kg/ha) and urea (150 kg/ha). Leucaena plantations were not fertilised. Rainfall and temperature data for Belmont are presented in Appendix 1.

The two experimental paddocks used on Brian Pastures contained either double rows of Leucaena with inter rows containing Rhodes and Green panic (*Panicum maximum* var. trichoglume), 4 ha at 3m spacing or approximately 15 ha Native Blue grass dominated pastures.

ii. Animals and management

The experimental protocol complied with the Australian Code of Practice for the care and use of Animals for Scientific Purposes (Aust. Gov. National Health and Medical Research Council, 2004) and was approved by the local Animal Experimentation and Ethics Committee (A13/2012).

ii.1 Belmont Research Station

Bos indicus cross steers [n = 60, initial mean (\pm sem) LW 296 \pm 5.2 kg] were randomly allocated to one of two management groups; Leucaena or grass fed. Animals were transferred to paddocks dominated by either established plantations of Leucaena or Rhodes grass pastures. Four days after steers were allocated to the Leucaena treatment, nine animals were drenched with 100 mL rumen fluid known to contain the mimosine degrading bacterium *Synergistes jonsei* (Allison *et al.*, 1992). At approximately 14 d intervals, to coincide with individual LW measurements, animals were rotated onto similar adjacent Leucaena or Rhodes grass paddocks, respectively, to ensure *ad libitum* intakes throughout the project. Individual animal LW data was used to calculate average daily gain (ADG, kg/d) by linear regression. All paddocks contained a dedicated water source. Herd scale methane measurements using open-path lasers were conducted on four occasions: March/April and June/July 2013; and March/April and August 2014. Determination of methane emissions at the herd scale were facilitated by confining animals to known areas (source areas) either adjacent to Leucaena paddocks

or within a Rhodes paddock for 5 h daily from approximately 0800 for up to 28 d for each group of animals. Confinement areas contained a water trough and measurements were conducted after the observed period of daily (am) grazing activity.

ii.2 Brian Pastures Research Station

Bos indicus and Composite weaner steers [$n = 60$, initial mean (\pm sem) LW 237 ± 3.4 kg] were randomly allocated to one of two management groups based on Leucaena or native grass pastures. Prior to the commencement of the project 10% of the original weaner herd was drenched with 100 mL rumen fluid known to contain the mimosine degrading bacterium *Synergistes jouseii*. Animals were transferred to paddocks dominated by either established plantations of Leucaena or Native Blue grass dominated pastures. At 28 d intervals, to coincide with individual LW measurements, animals were rotated into adjacent Leucaena or native grass paddocks, respectively, to ensure *ad lib* intakes. Individual animal LW data was used to calculate average daily gain (ADG, kg/d) by linear regression. All paddocks contained a dedicated water source. Herd scale methane measurements using open-path lasers were conducted in September/October 2013 only. Determination of methane emissions at the herd scale were facilitated by confining animals to a known area (source area) as described (2.1), however in this case only one source area was available. Consequently methane measures were conducted on alternate days for each grazing group.

iii. Pastures

iii.1 Sampling

At each site, and to coincide with methane measurements, pastures were assessed for total available biomass (kg DM/ha) by cutting twelve, 0.25 m^2 quadrats to nominal grazing height and species composition determined.

iii.2 Chemical composition of available forage

Collected forage biomass from each site was dried at 65°C in a forced draught oven to constant weight, weighed for estimating DM yields (kg DM/ha) and then ground, bulked and sub sampled for proximate analysis (dry matter, DM, organic matter, OM, crude protein, CP and fibre content). Pluck samples of leaf and stem (diam < 5 mm) were collected randomly across each Leucaena plantation at both sites for proximate analysis. Collected material was also sub sampled and placed onto dry ice, stored at -80°C and subsequently freeze dried for measurement of condensed tannin content.

Proximate analysis on forage samples was conducted by Symbio Alliance Eight Mile Plains QLD. In brief, moisture content (dry matter, DM) was determined by drying to constant weight, organic matter (OM) by ashing the dried samples at 550°C for 8 h in a muffle furnace. Acid detergent fibre (ADF) was determined without α -amylase treated neutral detergent fibre (NDF) using a FiberCap™ system (FC 221 FOSS Analytical A/S Denmark). Sodium sulfite was not used. aNDF was expressed exclusive of residual ash and total N content by micro combustion using a LECO autoanalyser. Crude protein (CP) was estimated as $6.25 \times \text{N}$.

Condensed tannin content in Leucaena forage was determined after grinding samples through a 1 mm sieve. Ground material was subjected to acid catalysed depolymerisation in the presence of phloroglucinol. Analysis of individual subunits (phloroglucinol analogues) was determined by a comparative absorbance method using HPLC. Grape skin tannin (4.73 mg) was depolymerised with the samples so that peaks could be identified by HPLC (Hixson *per. comm.*).

iii.3 Estimates of pasture intake

iii.3.1 Faecal NIRS

Dietary non-grass and grass proportions in the diet were estimated using near infrared reflectance spectroscopy (NIRS) as described by Coates and Dixon (2007).

To coincide with methane measurements at both sites rectal faecal samples from all animals were collected for NIRS (F.NIRS) to determine diet composition and intake (Coates and Dixon, 2007, 2008). Faecal samples were placed on ice directly after sampling and stored frozen until F.NIRs analysis. Thawed, dried faecal samples (approx. 2.5 g) were then ground through a 1 mm sieve (Cyclotec™ FOSS Analytical A/S Denmark), pre-dried in a 60 °C oven over 4 h and packed into ring cups. A scanning monochromator (spinning model 6500 with ISIScan™ FOSS Analytical A/S Denmark) generated spectral data for each sample to describe dietary N%, Diet CP%, DM digestibility (%), faecal $\delta^{13}\text{C}$, and estimates of DM intake (g/kg LW.d) using ISI software (Infrasoft International, Port Matilda, Penn) and calibration equations for a standardised tropical grass and Leucaena.

Dietary non-grass, assumed to be essentially Leucaena forage in this project, was calculated from predicted faecal $\delta^{13}\text{C}$:

$$\text{Non-grass (\%)} = (\text{faecal } \delta^{13}\text{C} - 13.5) \times 7.14 \quad (\text{Coates and Dixon, 2007})$$

iii.3.2 Determination of $\delta^{13}\text{C}$ by mass spectrophotometry

Samples of Leucaena browse (leaf and stem) collected from Belmont Research Station were submitted to CSIRO Canberra for the independent direct determination of $\delta^{13}\text{C}$. Samples were finely ground using a puck mill and dried overnight at 65°C. Dried samples were weighed into tin foil capsules (2.1±0.1 mg) for analysis by mass spectrophotometry. White wings flour (2.4 ± 0.1mg) was used as the reference.

The analysis of samples was performed using a Europa 20-20 isotope ratio mass spectrometer with an ANCA preparation system. The system included a combustion tube operated at 1000°C and a reduction tube at 600°C, a gas chromatograph (GC) and a mass spectrometer (MS). The sample was completely ignited in the combustion tube with a pulse of O₂. Helium was used as a carrier gas to transfer the gaseous sample, after flash point, from the combustion tube into the reduction tube which was packed with bright Cu metal so that the N and C fractions could be converted to N₂ and CO₂. The gas stream then passed through a GC and the N₂ and CO₂ components separated. The mass spectrometer measured the isotope ratios for the N₂ and CO₂ peaks sequentially in the gaseous sample.

iii.3.3 Chromic oxide

Controlled release rumen capsules delivering 632.2mg/d Cr₂O₃ in a soluble matrix were used to estimate pasture intake at the Belmont site during March/April 2014. Capsules were manufactured by CSIRO Chiswick, NSW. Twelve steers in each grazing group were dosed *per os* into the reticulum-rumen with one capsule and monitored for one hour to gauge retention of the devices. All animals retained their capsule and were returned to grazing for 5 d, so as to achieve equilibrium in the digesta before sampling.

No methane measurements were conducted during faecal sampling for intake estimates using the controlled release devices. Six days after introduction of the rumen capsules faecal samples (up to 400 g) were collected daily (19 d) from all steers sequentially either morning, mid-day or afternoon so as not to confound faecal marker concentration with sampling event or diurnal grazing behaviour. Faecal samples were placed on ice directly after sampling and stored frozen until dried at 70 °C in a forced draft oven to constant weight and milled through a 1mm sieve (Cyclotec™ FOSS Analytical A/S Denmark).

For the estimation of Cr in the faecal material samples were ashed to determine OM content. The residual material was subject to a sulphuric/ ortho-phosphoric acid digest prior to analysis using atomic absorption spectrophotometry to determine the Cr concentration ($\mu\text{g}/10 \text{ mL}$) in the digested samples. Cr concentrations were converted to mg/g DM equivalent and total forage intake (kg/d) calculated based on digestibility and faecal output.

iv. Rumen function

Rumen fluid samples of approximately 60 mL were collected *per os* using a stomach tube and manual pump from 20 animals in each grazing group on both sites to coincide with methane measurement periods throughout the project. All samples were immediately frozen on dry-ice and stored at -80°C until analysis.

iv.1 Volatile fatty acids

Volatile fatty acids were prepared by a modification of the technique of Moya *et al.* (2009). Briefly, frozen samples of strained fluid were thawed completely and aliquots of 1.5 mL were centrifuged at 16,500 g using a Prims R 24 microcentrifuge (Labnet International Inc., NJ, USA) at 4°C for 15 min. Supernatant aliquots (500 µl) were transferred into three GC vials, acidified with 10 µl of H₃PO₄ (~85% pure); and 50 µl of 4-Methylvaleric acid as an internal standard (11 mM solution).

Concentrations of the VFAs acetic, propionic, isobutyric n-butyric, isovaleric, n-valeric acid and caproic acid were determined by GC (Shimadzu GC-2014, Shimadzu, Tokyo, Japan) using a Zebtron™ ZB-FFAP column (30 m x 0.53 mm ID, Phenomenex, Torrance, CA, USA). The carrier gas was H₂ at 5 mL/min and separation of the acids was in 12.7 min/run. The injector and detector temperatures were 200°C and 230°C, respectively, while the column temperatures were initially 100°C for 2 min followed by a gradient of 15°C/min to 230°C (2 min hold). Peak detection and chromatogram integration were performed using the GC solution software (Shimadzu v 3.30.00).

iv.2 Rumen pH

Rumen fluid pH was measured immediately on collected liquor using a portable pH meter (TPS Aqua V0559).

iv.3 Microbial diversity and abundance

Sub samples of rumen fluid were collected for DNA based PCR/quantitative PCR, and 1.0 mL from each animal was mixed with 'RNA Later' for subsequent RNA extraction and analysis. In addition, rumen fluid (2.0 – 2.5 mL) from eight animals within each group was inoculated into 50% anaerobic glycerol bottles for microbial isolation.

v. Rumen metabolomics

Rumen fluid samples collected by stomach tube from cattle grazing a Leucaena (n = 8) or Rhodes grass pastures alone (n = 8) in June 2013 and July 2014 at Belmont Research Station were immediately frozen on dry ice and stored at -80°C until subjected to metabolite analysis.

v.1 Extraction of rumen fluid for GC-MS (Untargeted)

Fifty microliters of rumen fluid was transferred into an Eppendorf tube (2 mL). Cold methanol (100%, 150 µL) and a quantitative internal standard containing 1% [(¹³C₆-Sorbitol (0.5 mg/mL) and ¹³C₅-¹⁵N-Valine (0.5 mg/mL) was subsequently added. The sample was vortexed for 30 s and then left on ice for 30 min. The extracted fluid was then centrifuged at 13,000 rpm for 5 min at 23°C. An aliquot (100 µL) was transferred into a glass insert and dried *in vacuo* for subsequent TMS polar metabolite derivatisation. Extracted fluid samples were placed in a snaplock bag with silica gel prior to derivatisation for GC-MS analysis.

v.2 Polar metabolite TMS derivatisation

Dried samples were re-dissolved in 10 µL of 30 mg/mL methoxyamine hydrochloride in pyridine and derivatised at 37°C for 120 min with mixing at 500 rpm. The samples were then treated for 30 min with 20 µL N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and 2.0 µL retention time standard mixture [0.029% (v/v) n-dodecane, n-pentadecane, n-nonadecane, n-docosane, n-octacosane, n-

dotriacontane, n-hexatriacontane dissolved in pyridine] with mixing 500 rpm at 37°C. Each derivatised sample was allowed to rest for 60 min prior to injection.

v.3 GC-MS analysis

Samples (1 µL) were injected in splitless mode into a GC-MS system comprised of a Gerstel 2.5.2 autosampler, a 7890A Agilent gas chromatograph and a 5975C Agilent quadrupole MS (Agilent, Santa Clara, USA). The MS was adjusted according to the manufacturer's recommendations using tris-(perfluorobutyl)-amine (CF43). The GC was performed on a 30 m VF-5MS column with 0.2 µm film thickness and a 10 m Integra guard column (J & W, Agilent). The injection temperature was set at 250°C, the MS transfer line at 280°C, the ion source adjusted to 250°C and the quadrupole at 150°C. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. For the polar TMS metabolite analysis, the following temperature program was used; start at injection 70°C, a hold for 1 min, followed by a 7°C/ min oven temperature, ramp to 325°C and a final 6 min heating at 325°C.

Chromatograms and mass spectra were evaluated using the Agilent MassHunter Workstation Software, Quantitative Analysis, Version B.06.00/Build 6.0.388.0 for GC-MS. Mass spectra of eluting compounds were identified using the public domain mass spectra library of Max-Planck-Institute for Plant Physiology, Golm, Germany (<http://csbdb.mpimp-golm.mpg.de/csbdb/dbma/msri.html>) and the in-house Metabolomics Australia mass spectral library. All matching mass spectra were additionally verified by determination of the retention time by analysis of authentic standard substances. Resulting relative response ratios (area of analyte divided by area of internal standard, ¹³C₆-sorbitol) for each analysed metabolite as described in Roessner *et al.*, (2001). The data was also normalized in order to compare fold differences between groups. If a specific metabolite had multiple TMS derivatives, the metabolite with the greater detector response and improved peak shape within the dynamic range of the instrument was selected.

vi. Rumen microbial analyses

vi.1 DNA extraction

Genomic DNA was extracted from the thawed rumen samples using Cetyltrimethylammonium Bromide (CTAB; Qiagen, Hilden, Germany) by bead-beating followed by phenol–chloroform extraction, in duplicate for each sample. Rumen fluid samples (2 mL) were centrifuged inscrew-capped tubes at 13,000 rpm for 15 min and the supernatant removed. The pellet was resuspended in 600 µL of lysis buffer (2% CTAB, 100 mM Tris–HCl, 20 mM EDTA and 1.4 M NaCl), and 250 mg of zirconium beads (1: 1 mixture of 0.1 mm/1 mm; Biospec Scientific, Bartlesville, OK, USA). The samples were twice mixed in a bead-beating machine (FastPrep®; MPBiomedicals, Solon, OH, USA) at setting program 1 (60S). The samples were incubated for 20 min on 70°C heat block and inverted for 4 to 5 min, then centrifuged for 10 min at 13,000 rpm, and the dark supernatant removed to a new Eppendorf® tube (South Pacific Pty, Sydney NSW Australia). Chloroform/isoamyl alcohol (500 µL; 24: 1(v/v)) was added and the mixture was strongly shaken until completely cloudy, then centrifuged at 13,000 rpm for 10 min. The clean supernatant was removed to a new 2 mL Eppendorf® tube without any white layer material, phenol/chloroform/isoamyl alcohol (24: 24: 1(v/v)) was added into tubes and vortexed to form a white emulsion to separate the aqueous and organic layers, and spun again for 10 min at 13,000 rpm. The upper aqueous layer was transferred to a new tube and the DNA precipitated with isopropanol (0.8 vol). After standing at -80°C in the freezer for 1 h, thawed at room temperature, the DNA pellet was recovered by centrifugation at 10,000 rpm for 25 min. The DNA pellet was washed with 70% cold ethanol (500 µL) and then air-dried for 10 min. The DNA extracts were dissolved in 200 µL EB buffer and DNA yield was quantified using a NanoDrop ND-1000 Spectrophotometer (Nyxor Biotech, Paris, France). Because most of the concentrations are higher than 1000ng/µL, the DNA extracts were diluted 10 times and 50 times in EB buffer prior to qPCR reactions and 1µL of the diluted DNA solutions were used as templates.

vi.2 Next generation sequence analysis

High throughput sequencing platforms and barcode “pyrotagging”, phylogenetic based methods targeting the 16S rDNA gene were used to deeply characterise the bacterial and archaeal populations present in the rumen of approximately 20 cattle grazing Rhodes grass and 15 cattle grazing Leucaena/Rhodes grass at the Belmont and 20 cattle grazing native mixed pasture and 20 cattle grazing Leucaena/native pasture at Brian Pastures. 16S rDNA gene pyrotagging was performed using modified universal bacterial primers (515F and 806R). Specific sequences matching the Illumina Miseq sequencing adaptor P5 were added to the 515f primer, while the P7 adaptor was added to the 806R. In addition, a dual index primer approach was used with index primers attached to both the forward and reverse primers, allowing for a multiplexing of 384 samples from 24 unique forward indexes and 36 reverse indexes. Each individual DNA sample was amplified using a unique index combination. After amplification, products were visualised by performing gel electrophoresis. Product quantities were calculated and an equal molar amount of each product was pooled. The pooled products were run in a 1.5 % agarose gel and the product gel extracted and purified prior to submission for Illumina Miseq pyrosequencing.

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 and further analysis using R with the Ade4, phyloseq (Thioulouse *et al.*, 1997, McMurdie and Holmes 2013).

vi.3 Quantitation of microbial populations

Quantitative PCR (qPCR) assays were performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Assays were set up using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Optimisation of assay conditions was performed for primer, template DNA and MgCl₂ concentrations. An optimal primer concentration of 300 nM and a final MgCl₂ concentration of 3 mM were finally chosen for the assay under 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. Fluorescence detection was performed at the end of each denaturation and extension step. Amplicon specificity was performed via dissociation curve analysis of PCR end products by raising the temperature at a rate of 1°C / 30 sec from 60°C to 95°C. Total microbial rumen DNA was diluted to 1:10 prior to use in real time PCR assays to reduce inhibition. Each reaction (standard curves and samples) was conducted in quadruplicate.

Relative qPCR assays were performed to measure the abundance of *Ruminococcus albus*, *R. flavefaciens*, *Fibrobacter succinogenes* and total protozoa relative to abundance of total bacteria using specific primers (Table 1). The $2^{-\Delta\Delta CT}$ method was used for determination of relative abundance of microbial populations, where CT value represents the threshold cycle at which amplified product was first detected in qPCR amplification. ΔCt is difference in CT value of the target gene from the Ct value of total rumen bacterial 16S rDNA as a reference gene in the rumen.

Table 1. Primers used for the quantification of rumen bacteria using real-time PCR assay

Target Species	Forward/Reverse	Primer sequences (5'---3')	Reference
Total bacteria	F	CGGCAACGAGCGCAACCC	Denman and McSweeney 2006
	R	CCATTGTAGCACGTGTGTAGCC	
Protozoa	F	GCTTTCGWTTGGTAGTGTATT	Sylvester <i>et al.</i> 2004
	R	ACTTGCCCTCYAATCGTWCT	
<i>Fibrobacter succinogenes</i>	F	GTTCGGAATTACTGGGCGTAAA	Denman and McSweeney 2006
	R	CGCCTGCCCCTGAACTATC	
<i>Ruminococcus flavefaciens</i>	F	CGAACGGAGATAATTTGAGTTTAC	Denman and McSweeney 2006
	R	CGGTCTCTGTATGTTATGAGGTATTACC	
<i>Ruminococcus albus</i>	F	CCCTAAAAGCAGTCTTAGTTCG	Koike and Kobayshi 2001
	R	CCTCCTTGCGGTTAGAACA	

vii. Herd scale methane measurements

vii.1 Field instrumentation

Four independent herd scale methane measurement campaigns were conducted at Belmont and one at Brian Pastures Research Station. While actual implementation varied slightly (Table 2), field instrumentation at each site was similar.

Animals were confined to measurement compounds (~620m², source areas) on a daily basis that had no major obstruction to wind flow (Figure 1). Water was freely available, and animals were returned to paddocks after approximately 5 h measurements. Measurements were restricted to between 0800 and 1500 due to high occurrence of unsuitable atmospheric conditions in the night and early morning, and the requirement to return cattle to the paddocks for grazing in the afternoon.

Open path lasers (OPLs) (GasFinder2.0, Boreal Lasers Inc., Spruce Grove, AB, Canada) were used to measure methane emissions upwind of each compound. These lasers were mounted on a Digital Scanning Motor (PTU D300, Directed Perception Inc., Burlingame, CA) that permitted multiple measurements of CH₄ concentration sequentially along two different paths to accommodate wind direction changes relative to source area. Lasers interfaced with control software (GasView MP, Boreal Laser Inc.) and allowed a one minute measurement period on one side of the compound before rotating to measure the other side for one minute. The system also monitored laser light levels and optimised laser position to improve light when levels were outside pre-set limits (3000 and 12,000, no units). A third (stationary) laser was used to measure background methane emissions. This laser was

placed upwind, more than 100 m from either compound. The OPL and PTU systems were powered by two 12 V batteries, which were connected to solar panels (2 x 120 W) to recharge the batteries during the day. The third OPL was powered with a 12V battery connected to a solar panel (80 W). Retro reflectors (Boreal Lasers) reflected the signal back to the lasers on all paths. Lasers recorded line averaged methane concentrations (ppm) for each path once per second.

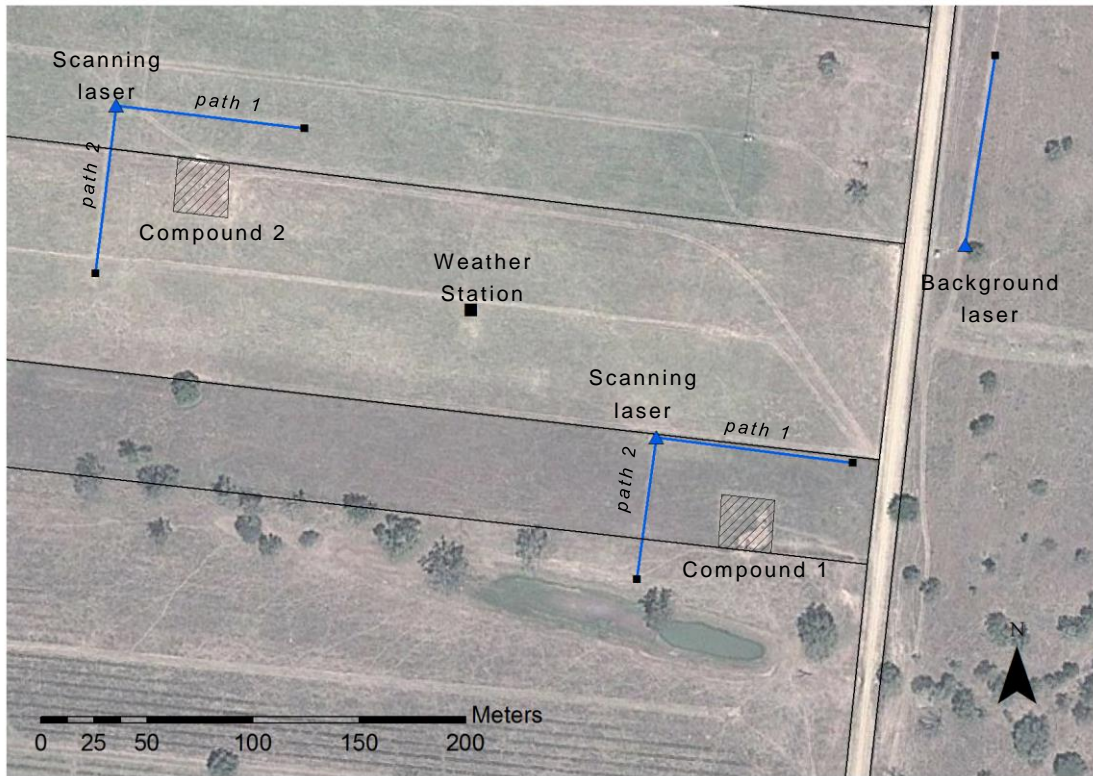


Figure 1. Map of Belmont study site indicating source areas used to determine herd scale methane measurements for cattle grazing *Leucaena* or *Rhodes* grass pastures. A sonic anemometer was fitted to the weather station. Open path lasers (\blacktriangle), retro reflectors (\blacksquare). Black lines indicate paddock boundaries. Background image: Google, DigitalGlobe (2015).

A sonic anemometer (CSAT3, Campbell Scientific Inc.) and data logger (CR1000, Campbell Scientific Inc.) were mounted on a weather station mast midway between the two source areas with the sonic head facing into prevailing winds. For Belmont trials this was an offset of 90° from true north and for Brian Pastures 180° . The CSAT3 was used to measure atmospheric conditions including temperature and wind parameters. Following McGinn *et al.* (2015), average values of wind speed, temperature and associated variance and cross products were recorded over 10 min. Data from a pressure sensor, temperature and humidity probe, wind vane and three-cup anemometer, and three shielded type T thermocouples set at varying heights were also logged.

Position of all equipment for the Belmont trials was measured using an Ashtech Promark 100 GPS in March 2013. Post-processing data against a static base and the TOW02 reference station (part of the International GNSS Service network) provided sub-meter accuracy (~ 0.3 m). Distance between points (Table 2) could then be found using maps constructed in WindTrax (Thunder Beach Scientific). For consistency all equipment at the Belmont site was set-up at the same location in all trials. Sonic anemometer, pressure sensor and laser heights varied slightly across trials (Table 2).

Prior to each experimental period OPLs were run side by side to evaluate between laser biases. As trials were conducted during daytime calibration was also carried out during daytime. Data collected was used to correct CH₄ measurements for all OPLs against an arbitrarily chosen reference laser (OPL 1042) (Table 3). In addition, for the Belmont data set lasers paths were cross-calibrated with each other using an in-situ approach (using data taken during measurement periods with lasers in their actual measurement positions). They were selectively cross-calibrated against the background laser during restricted wind directions. The idea being that with our setup there will be periods when the wind direction exposes different laser path combinations to the same “fresh-air” concentration (even with cattle in the paddock):

- Wind from 0 to 80°: the background and north paths (path 1) should give the same background CH₄ concentration,
- Wind from 185 to 210°: background and west paths (path 2) should give the same concentration.

Logged data was corrected for temperature and pressure following manufacturer relationships. Measurement periods (10 min) having a wind direction within the above ranges were used to develop multipliers to force the concentrations of various laser paths to match that of the standalone laser. In principle this is a good approach as it eliminates systematic measurement errors due to errors in the measured laser path lengths, the possibility of errors due to different laser signal levels on the different paths and reflector differences. In a final step, all the cross-calibrations were adjusted assuming Laser 1042 was the correct standard.

The procedures and field instrumentation employed in the Brian Pastures trial were similar to those used at Belmont with minor logistical changes (Table 2). Only one compound was used (Figure 2) so, measurement days were alternated between the two grazing groups. Further details of each trial and description of variations are detailed in Table 4.

Table 2. Field instrumentation for direct measures of herd scale methane emissions. Sensor heights and locations (¹ path lengths consistent for all Belmont measurement periods)

	CSAT3 height (m)	Pressure sensor (m)	Scanning Laser (m)	Background laser (m)	Path lengths (m)				
					Compound 1 Path 1	Compound 1 Path 2	Compound 2 Path 1	Compound 2 Path 2	Background
Belmont									
Mar/Apr 2013	2.88	1.75	1.60	1.60					
Belmont									
Jun/Jul 2013	2.88	1.72	1.60	1.70	93.3 ¹	66.8 ¹	89.3 ¹	79.5 ¹	90.1 ¹
Belmont									
Mar/Apr 2014	2.97	1.60	1.60	1.70					
Belmont									
Aug 2014	3.05	1.60	1.60	1.70					
			<u>Laser height (m)</u>			<u>Path length (m)</u>			
Brian Pastures Sept/Oct 2013	2.78	1.70	North	East	South	North	East	South	
			1.7	1.6	1.7	81.5	83.6	89.7	

Table 3. Correction coefficients¹ applied for each methane measurement period using open path lasers.

		LASER ID			
		1042	1034	1012	1013
Belmont Mar/Apr 2013	Correction factor	1	1.0424	1.1466	
	SD ²	0	0.0225	0.0227	
	n ³		77	196	
Belmont Jun/Jul 2013	Correction factor	1	1.0793		
	Standard deviation	0	0.0150		
	n		226		
Belmont Mar/Apr 2014	Correction factor	1	1.0651	0.9908	1.0313
	Standard deviation	0	0.0298	0.0208	0.0225
	n		393	492	535
Belmont Aug 2014	Correction factor	1	1.1080		
	Standard deviation	0	0.0363		
	n.		476		
Brian Pastures Sept/Oct 2013	Correction factor to 03/10/15 ⁴	0.9310	1		
	n		78		
	Correction factor from 03/10/15	0.8997	1		
	n		343		

¹Not corrected for temperature and pressure, ²Standard deviation, ³ number of 10 min averages; ⁴Laser blown over in high winds and recalibrated.

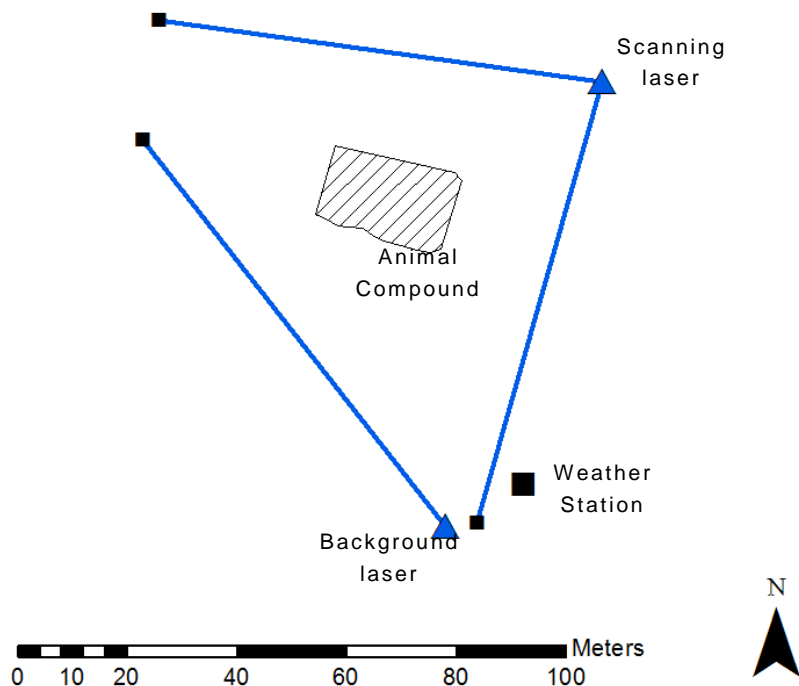


Figure 2. Map of Brian Pastures study site indicating source area, weather station with sonic anemometer and three laser paths. Open path lasers (▲), retro reflectors (■).

Table 4. Number of measurement days, animals used and comment for each methane measurement campaign 2013/2014

	Measurement days		<i>n</i>		Comments
	Leucaena	Pasture	Leucaena	Pasture	
Belmont Mar/Apr 2013	14	12	30	30	<i>No change from described procedures.</i>
Belmont Jun/Jul 2013	18	16	29	30	<i>Due to availability of only 1 scanning laser the laser was rotated between compounds. Leucaena animals were measured first (15 d). Pasture animals then measured (14 d). For the remaining time measurements were in one compound and groups confined on alternate days.</i>
Belmont Mar/Apr 2014	9	9	29	30	<i>Due to availability of only one scanning laser the laser was rotated between compounds. Leucaena steers measured first (4 d), later pasture animals measured (4 d). An additional laser became available; both compounds measured simultaneously</i>
Belmont 2014 August		18		30	<i>Only pasture group available for measurements. Leucaena steers slaughtered for carcass characteristics.</i>
Brian Pastures Sept/Oct 2013	11	10	29	30	<i>Single compound, animal groups alternated for measurements</i>

vii.2 Dispersion model

Atmospheric and laser data was processed to predict 10min average CH₄ emissions using the WindTrax software tool (WindTrax version 2.0.8.8, Thunder Beach Scientific, www.thunderbeachscientific.com) and a backward Lagrangian stochastic (bLS) model (McGinn *et al.*, 2008). In the simulations modelled 50,000 trajectories were used and confined cattle treated as a uniform area source at 0 m elevation. McGinn *et al.* (2015) showed no significant difference in using a 0 m elevation compared with a 0.5 m elevation (chosen as an approximate animal head height). In WindTrax the atmospheric pressure was calculated from the elevation. Background (upwind of the source and/or unaffected by point-source dispersion plumes) CH₄ concentrations were predicted by the WindTrax model. The WindTrax-simulated plume is used to predict an emission-to-concentration relationship (Q/C)sim. This simulated relationship is used with measured 10min CH₄ concentrations (OPL multiple paths) to infer the daily herd CH₄ emission. For each source area, the mean animal CH₄ emission (g/d) was calculated by dividing the hourly source area emissions by the number of animals in the source area and multiplying by 24. The bLS model is described further by Flesch *et al.* (2004).

vii.3 Data management

Daily methane concentration files were merged into a single file for each laser using SAS (SAS Inst., 1999). Methane concentrations were calculated using laser (OPL) recorded ppm values and individual path lengths. These values were standardized by multiplying against the appropriate correction coefficient (Table 3). Concentration data was converted to 10 min averages for each path during measurement periods. CSAT3 anemometer and other weather data was merged into a single file and statistical parameters including surface roughness (Z₀), Monin-Obukhov length (L) and friction velocity (u*) calculated.

Averaged meteorological and OPL datasets were used as input files to WindTrax (WindTrax version 2.0.8.8, Thunder Beach Scientific) with relative spatial data provided by generating WindTrax maps for each site. For Belmont data independent models were used for each source area. The WindTrax model used weather conditions and laser CH₄ ppm values to estimate 10 min averaged methane flux

(g/h) originating from each source area. The resulting values were multiplied by 24 h/animal number and the average of all values taken for each group of animals to obtain a mean estimate in g/head per day. The WindTrax model also calculated values for Z_0 , L and u^* . These were compared with SAS calculated values to verify input data.

Data was filtered for periods when the animals were not confined to source areas, and for highly unsuitable weather conditions (including fog and rain). Atmospheric and laser parameters were used to remove further unreliable periods as described by Loh *et al.* (2008) and Flesch *et al.* (2007).

The following criteria were used to remove potentially inaccurate or unrepresentative concentration measurements for Belmont observations (2013 and March 2014 only):

- Laser observations < 50% of potential. This criterion eliminates any period where the laser observations corresponded to less than 50% of the potential measurement period (< 5 min of a 10 min observation).
- $R^2_{\min} < 98$. R^2 is a parameter given by the laser for each observation, and relates to the quality of the concentration measurement from the laser. We eliminated all data where R^2 was less than 98. This eliminated periods when the spectrum from the reference cell did not match that from the sample spectrum.
- $4,000 < \text{Light Level} < 12,000$. Light level is a laser operating parameter related to the signal level of the returning laser beam. The manufacturer advises that concentration measurements may be inaccurate if the signal level falls outside this range.

Not all observation periods are expected to give good emissions estimates. The following criteria were used to remove error-prone periods. These criteria have been used in previous studies.

- $u^*_{\text{thres}} = 0.05$ m/s. This criterion removed low wind speed periods when the friction velocity u^* is less than a threshold u^*_{thres} . We used a lower threshold for u^* than many earlier studies in order to increase our dataset (as we accepted low wind speed periods). Flesch *et al.* (2014) concluded that a low u^*_{thres} (0.05 instead of 0.15 m/s) introduces outliers in the calculated emission data set, but there is not much change in the overall average accuracy.
- $|L|_{\text{thres}} = 2.0$ m. This criterion excluded periods when the atmospheric stratification is extreme -- when the absolute value of the Obukhov length L falls below the threshold $|L|_{\text{thres}}$ (Flesch *et al.*, 2004).
- $Z_0_{\text{thres}} = 0.25$ m. This criterion used the calculated surface roughness Z_0 to indicate periods when wind conditions did not conform to the meteorological assumptions in WindTrax (Monin Obukhov similarity). For sites with a plant canopy we expected Z_0 to fall within the broad range of 5 to 25% of the canopy height. Here a Z_0 above $Z_0_{\text{thres}} = 0.25$ m indicated wind conditions that violated WindTrax assumptions.
- $\text{tdcov}_{\text{thres}} = 0.95/1.00$. This important criterion is based on the fractional coverage of the downwind laser measurement "footprint" over the source area (Flesch *et al.*, 2007). For some wind directions the plume from the cattle paddock only "glanced" the path of the lasers. This was a concern: the plume edge carries greater model uncertainty, since extreme trajectories at the plume margin are less predictable. Because the laser footprint only covers a portion of the source area (which may or may not contain animals in any particular observation), it can lead to poor estimates of average paddock emissions. With the footprint only covering a portion of the source area slight errors in the wind observations (particularly wind direction) can introduce dramatic errors in the emission estimates. To avoid these problems we removed periods where the fractional laser footprint tdcov covered less of the source area than the threshold $\text{tdcov}_{\text{thres}}$ of either 0.95 or 1.00. We tended to use $\text{tdcov}_{\text{thres}} = 1.00$, but due to limited data periods we included results using a relaxed threshold of 0.95 for March 2014 data.

Resultant output data obtained from Brian Pastures was retained on the basis of the following criteria:

- (1) Laser light (unit less number) and R^2 values within the limits: $4000 < \text{Laser light level} < 12000$ and $R^2 \geq 0.98$. Data within 1 min following $R^2 < 0.98$ or 1 min following a low light level error message was also automatically deleted to allow the laser time to return to a stable state.
- (2) Standard deviation of light level < 1000 within a 10 min period.
- (3) Suitable atmospheric conditions: $Z_0 < 0.25\text{m}$, $u^* \geq 0.05 \text{ m/s}$ and $|L| \geq 2\text{m}$. Z_0 is surface roughness, using a limit of 0.25m removed unrealistic values. L and u^* limits avoided very stable and low wind conditions while keeping the maximum amount of data possible (Flesch *pers. comm.*, 2014). Older limits of 0.15 and 10 were used for u and $|L|$ respectively (McGinn et al., 2011). It is unlikely that this difference significantly affected results.
- (4) Belmont: $-45^\circ < \text{wind direction} < 225^\circ$ where sonic anemometer was orientated 90° .

Brian Pastures: wind direction $< 45^\circ$ or wind direction $> 135^\circ$ where sonic anemometer was orientated 180° . This was to discard sonic data collected when the wind was coming from behind the sonic, which may be subject to high flow distortion error (Loh *et al.*, 2008).

- (5) Standard deviation of wind direction $< 45^\circ$ within 10 min blocks.
- (6) Fraction of source area covered in Windtrax model ≥ 0.95 .
- (7) A background laser $n > 40$ was used. The laser averaging function was used on the background laser giving a maximum $n = 75$. Due to unsuitable conditions and integrity of background laser data during the Brian Pastures measurement period the final number of 10 min averages was 35 and 45 for grass and Leucaena grazing animals, respectively.

Final data interrogation excluded any values greater than one standard deviation (SD) from the mean 24 h emission value. This removed any outliers and values where flux $< 0 \text{ ug/s}$ (Loh *et al.*, 2008).

Additional filtering and data processing was applied to the initial data set to determine the effect on flux values to further identify the inconsistencies in relative emissions between campaigns. Emissions were calculated using WindTrax (inverse-dispersion technique). Not all observation periods allow for good calculations and filtering criteria were used to eliminate periods in which: 1) the concentration measurements were believed to be inaccurate or unrepresentative; and 2) when the WindTrax dispersion calculations were potentially inaccurate. Data and measurement approaches will continue to undergo refinements by Dr Tom Flesch who is the international expert on open path laser measurements in grazing systems and is currently visiting CSIRO as a McMaster Fellow.

viii. Carcass parameters and MSA reporting

A subset of animals from each grazing group was identified for slaughter to determine carcass characteristics and MSA scores. In June 2014, 14 steers from the Leucaena grazing group with a mean (\pm sem) LW of $687 \pm 10.6 \text{ kg}$ were transported from Belmont Research Station to a commercial abattoir (TEYS Australia Pty Ltd. Biloela, Queensland) and slaughtered under industry practice. Carcass characteristics (carcass weight, P8 fat depth) were measured prior to chilling. Carcass pH and temperature was also measured.

AUS-MEAT marbling score, eye muscle area (EMA) and subcutaneous fat colour were measured on the *M. longissimus dorsi* (LD) 24 h post slaughter according to the Australian Bovine Carcass Assessment Scheme (AUS-MEAT 1998). Cattle were classed as MSA Grass fed ox and steer on Grid 025-14.

In September 2014 the remaining steers from both grazing groups were transported from Belmont Research Station to a commercial abattoir (JBS Australia Pty Ltd, Nerimbera Queensland) and slaughtered under commercial practice. This slaughter included 14 steers from the Rhodes grass group with a mean (\pm sem) LW of 526 ± 7.7 kg. Carcass characteristics and MSA scores were recorded, as described, for all animals.

ix. Whole farm modelling

To scale the animal-level measurements to the whole-farm level, a farm enterprise model was used to generate herd structures and an emissions model was used to estimate GHG, assuming pastures were dominated by Rhodes grass or Leucaena, with Rhodes grass representing the baseline.

The effects of Leucaena on GHG emissions, production and profitability at the whole farm level for a property in northern Australia was modelled as described by Harrison *et al.*, (2015). Relative differences in methane emissions of up to 25 % was used to inform the modelling analyses based on estimated methane emissions for each measurement period conducted on Belmont Research Station from cattle on either Rhodes grass or Leucaena.

In brief, the effects of finishing cattle on Leucaena based pastures at the whole farm level was modelled using BreedCowPlus (V6.0) to generate herd structures equivalent to a property of 267 ha grazing 400 AE at 16 months or older with baseline stocking rate of 1.5 AE/ha. The Beef Greenhouse Accounting Framework which uses Australian National Greenhouse Inventory methods prescribed by the DCCEE (2012) was used to estimate emissions. Greenhouse gas emissions included those from livestock (enteric and manure), urine, dung, and indirect emissions via ammonia volatilisation. To contrast Leucaena with a baseline property with Rhodes grass, three equivalent leucaena scenarios were modelled by matching (1) annual average stocking rate, (2) liveweight turnoff or (3) net farm emissions with that of the baseline, assuming that all animals had access to Leucaena. A fourth scenario was also modelled where only steers had access to leucaena. To maintain average annual stocking rate or liveweight turnoff, scenarios 1 and 2 carried 5 and 12% less cattle than the baseline because animals were of greater liveweight. Carbon offset income was determined relative to baseline data assuming two C prices (\$10 and \$23 /t CO₂-e) to represent current and historical prices and provide an indication of gross margin sensitivity to carbon price.

3. Results

Sixty *Bos indicus* steers continually grazed either a Rhodes grass dominated pasture (n=30) or one containing an established Leucaena plantation (n=30) on Belmont Research Station for 608 d. Herd scale methane measurements using open-path lasers were conducted on four occasions; March/April and June/July 2013, and March/April and August 2014. To coincide with methane measurements, available biomass was determined and sampled, rumen fluid collected from animals for fermentation and microbial analysis and intakes determined using F.NIRS or, in April 2014, an indigestible rumen marker. Liveweight was measured throughout the project at regular intervals. Rainfall events (mean monthly > 175 mm) throughout the study period on Belmont Research Station alleviated the requirement for irrigation (Table A2). Mean annual rainfall, 2000 to 2014, was 758 mm, with total rainfall over the duration of the study exceeding the 14 year mean in both years. Monthly mean minimum and maximum temperatures are shown in Table A2.

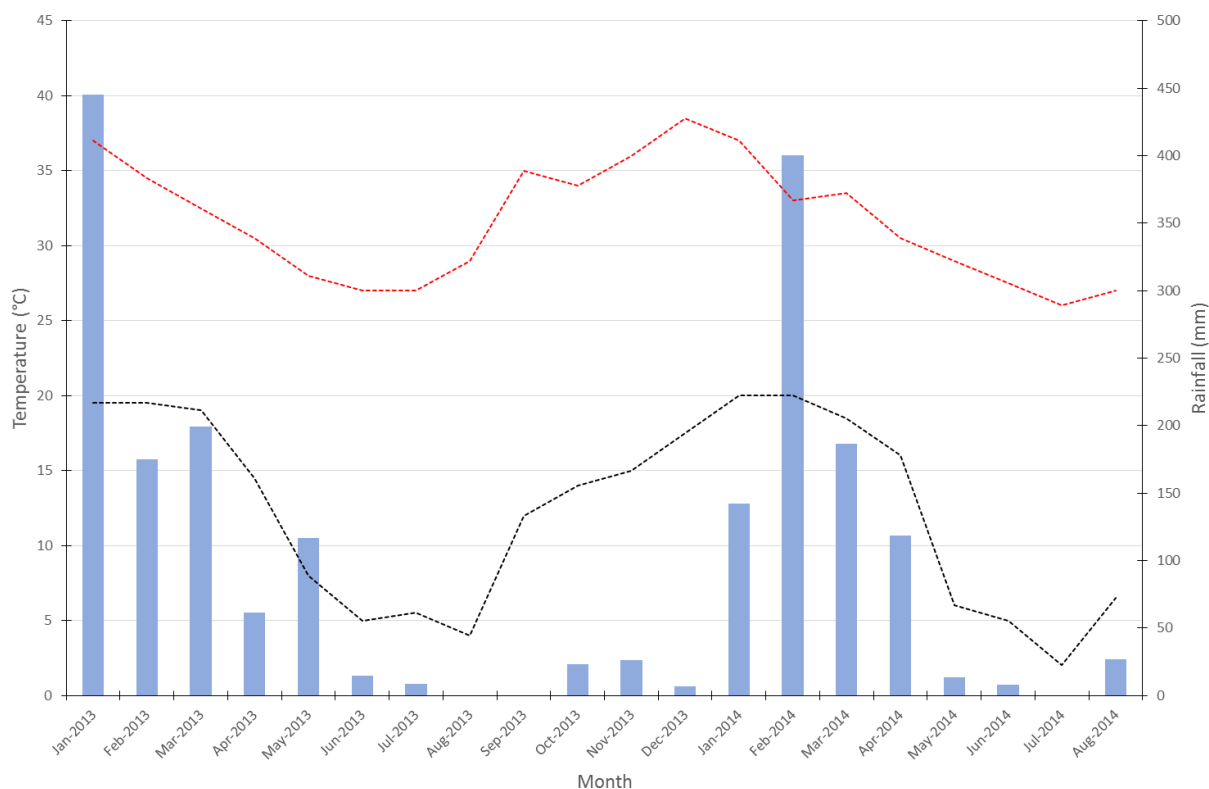


Figure A2. Mean monthly rainfall (■), minimum (---) and maximum (---) temperatures recorded for Belmont Research Station, January 2013 to August 2014.

Sixty *Bos indicus* and composite steers continually grazed either a dryland *Dicanthium* sp. dominated pasture (n=30) or one containing an established *Leucaena* plantation (n=30) on Brian Pastures for 192 days. In contrast to conditions on Belmont, less than 400 mm of rainfall was recorded on Brian Pastures prior to the commencement of the project with only an additional 176 mm during the study period. Mean annual rainfall over 58 years prior to the study period was 704 mm. Consequently the property was drought declared and destocked. Only one methane measurement and associated sampling was conducted on this site. Mean annual (1968- 2007) minimum and maximum temperatures for Brian Pastures was 14.4 °C and 28.1 °C, respectively.

ACTIVITY 1

Determine the reductions in methane emissions of cattle browsing *Leucaena* plantations in north Queensland compared with grass pasture fed animals

1.1 Composition and nutritive value of pastures

1.1.1 Belmont Research Station

Nutritive value of the available biomass for the four campaigns (2013-2014) on Belmont are provided in Table 5 and 6. Throughout the project grass dominated pastures consisted of >95% Rhodes grass (*Chloris gayana*) with some Sabi grass (*Urochloa mosambicensis*) (<5%) and small incursions of

Siratro (*Macroptilium atropurpureum*). *Leucaena* plantations contained a mix of Rhodes grass, Sabi grass and Green Panic in the inter rows. Mean total organic carbon (%) determined by MS was 44% and 48% for grass pasture and *Leucaena* browse samples collected in 2013, respectively.

Faecal NIRS (F.NIRS) results predicted diet quality (N%) to be higher for steers grazing *Leucaena* plantations compared with steers grazing Rhodes grass pastures throughout 2013 and 2014. Mean (±

sem) intakes estimated from F.NIRS were also generally higher for steers grazing Leucaena compared with those on Rhodes grass pastures (Table 7). Similarly, the proportion of non-grass in the diet, assumed to be Leucaena browse, ranged from 9.5 to 29.9% for steers with access to Leucaena plantations compared with steers on Rhodes grass pastures where the non-grass proportion was < 15%, and for April 2013 and August 2014 < 5% of the diet. Approximately 22% of the diet for steers managed in Leucaena plantations was found to be non-grass and regarded as Leucaena browse. Across 2014/15 the diet of Leucaena finished steers was 22:78 % compared with steers grazing Rhodes grass pastures where the proportion was 8:92 % for non-grass : grass, respectively. Faecal $\delta^{13}\text{C}$ analysis by MS suggested a diet composition of 6:94% (non-grass: grass) for steers grazing Rhodes pastures and aligns with F.NIRS estimates. However, steers grazing Leucaena browse were estimated to be consuming a diet consisting of 27:73 % (non-grass: grass) and higher than results indicated by F.NIRS.

Mean (\pm sem) daily grazing intakes estimated using slow release Cr_2O_3 capsules over 9 to 11 d for seven steers per group, where continuous data was available, were found to be 7.6 ± 0.28 kg/d and 8.8 ± 0.31 kg/d (DM basis), equivalent to 13 ± 0.05 g/kg LW and 16 ± 0.6 g/kg LW for Leucaena and Rhodes grazing steers, respectively. By comparison, F.NIRS estimates were 22.4 ± 0.19 and 21.1 ± 0.20 g/kg LW for steers grazing Leucaena or Rhodes grass pastures, respectively.

Table 5. Dry matter (DM), organic matter (OM), nitrogen (N), neutral detergent fibre (NDF) and acid detergent fibre (ADF) content of available forage diets on Belmont and Brian Pastures Research Stations for each methane measurement campaign 2013

	<u>Belmont Research Station</u>				<u>Brian Pastures</u>	
	March/April		June/July		October	
	Pasture ¹	Leucaena	Pasture ¹	Leucaena	Pasture ²	Leucaena
DM, g/kg	931	910	939	930	942	924
OM ³	895	927	912	919	877	909
N	11.8	60.3	8.0	38.6	3.0	36.3
NDF	692	225	728	377	766	222
ADF	389	189	458	247	588	160

¹Predominantly *Chloris gayana*, some *Urochloa mosambiensis*; ²Predominantly Leucaena; ³g/kg DM basis unless otherwise indicated

Table 6. Dry matter (DM), organic matter (OM), nitrogen (N), neutral detergent fibre (NDF) and acid detergent fibre (ADF) content of available forage diets on Belmont Research Station for methane measurement campaigns in 2014

	<u>March</u>		<u>August</u>	
	Pasture ¹	Leucaena	Pasture ¹	Leucaena ²
DM, g/kg	956	936	938	–
OM ³	918	929	931	–
N	6.9	48.1	7.6	–
NDF	739	286	748	–
ADF	611	241	431	–

¹Predominantly *Chloris gayana* some *Urochloa mosambiensis*, ²No Leucaena present; ³g/kg DM basis unless otherwise indicated

Table 7. Mean (\pm sem) F.NIRS estimates of Dietary N, faecal N, DM digestibility, digestible DM intake, DM intake and proportion of non-grass and grass in the diet for steers grazing pasture with or without *Leucaena*; Belmont Research Station 2013 and 2014

	<u>March 2013</u>	<u>June 2013</u>	<u>March 2014</u>	<u>August 2014</u>
	<u><i>Leucaena plantation</i></u>			
<i>N</i>	25	26	26	-
Dietary N (%)	1.9 \pm 0.05	2.1 \pm 0.04	2.1 \pm 0.04	-
Faecal N (%)	1.9 \pm 0.03	1.9 \pm 0.03	1.8 \pm 0.03	-
DM digestibility (%)	59 \pm 0.44	64 \pm 0.3	64 \pm 0.03	-
Digestible DM intake (g/kg LW.d)	16.7 \pm 0.32	15.2 \pm 0.28	13.1 \pm 0.19	-
DM intake (g/kg LW.d)	26.2 \pm 0.32	24.8 \pm 0.34	22.4 \pm 0.19	-
Non-grass in diet (%)	9.5	26.5	29.9	
Grass in diet (%)	90.5	73.5	70	
	<u><i>Pasture (Rhodes)</i></u>			
<i>N</i>	26	23	26	28
Dietary N (%)	1.3 \pm 0.05	1.3 \pm 0.04	1.2 \pm 0.03	0.7 \pm 0.03
Faecal N (%)	1.4 \pm 0.02	1.4 \pm 0.03	1.3 \pm 0.01	1.1 \pm 0.01
DM digestibility (%)	57 \pm 0.26	60 \pm 0.26	61.5 \pm 0.18	53.8 \pm 0.25
Digestible DM intake (g/kg LW.d)	14.3 \pm 0.16	11.5 \pm 0.22	11.8 \pm 0.18	6.9 \pm 0.16
DM intake (g/kg LW.d)	23.4 \pm 0.19	19.7 \pm 0.28	21.1 \pm 0.20	14.1 \pm 0.24
Non-grass in diet (%)	3.7	14.5	10.2	4.5
Grass in diet (%)	96.3	85.5	89.8	95.4

1.1.2 Brian Pastures Research Station

Nutritive value and measure of available biomass on Brian Pastures was determined once in September 2013. *Leucaena* browse (923 g/kg DM) was (g/kg DM) of ash, 91; crude protein (CP) 226.7; neutral detergent fibre (NDF) 222; acid detergent fibre (ADF) 159. Standing pasture (943 g/kg DM) consisting of mainly *Dicanthium sericeum* and *D. aristatum* (70%), *Bothriochloa bladhii* and some *P. maximum* var. *trichoglume* (<10%) was (g/kg DM) of ash, 124; crude protein (CP) 18.7; neutral detergent fibre (NDF) 766; acid detergent fibre (ADF) 587.

Mean total organic carbon (%) determined by MS was 37% and 45% for native grass pasture and *Leucaena* browse samples collected in September 2013, respectively.

F.NIRS data indicated that steers ($n=25$) grazing the available dryland *Leucaena* were consuming a diet with dietary N, 1.4 \pm 0.04 % and DM digestibility 58.3 \pm 0.37 % with Digestible DM intakes of 11.8 \pm 0.25 g/kg LW.d equivalent to a DM intake of 21.3 g/kg LW.d. Faecal N was estimated to be 1.8 \pm

0.05 %. Estimated mean diet composition (F.NIRS) suggested that up to 57 % of the diet was non grass and 43 % was grass (sem, 2.68%). Steers (n= 26) grazing the available native grass pasture were consuming a diet with dietary N, 0.9 ± 0.03 % and DM digestibility 55.6 ± 0.29 % with Digestible DM intakes of 8.9 ± 0.16 g/kg LW.d equivalent to a DM intake of 22.1 g/kg LW.d. Faecal N was estimated to be 0.84 ± 0.01 %.

Estimated mean diet composition (F.NIRS) suggested that up to 17 % of the diet was non grass and 83 % was grass (sem, 2.67%). Faecal $\delta^{13}\text{C}$ analysis by MS also suggested a diet composition of 17:83% (non-grass: grass) for steers grazing established native pastures. However, steers grazing *Leucaena* browse were estimated to be consuming a diet consisting of 39:61 % (non-grass: grass) and were lower than results indicated by F.NIRS.

1.2 Tannin content of *Leucaena* browse

The tannin content and composition of the *Leucaena* browse sampled from both sites has been characterised by the methods developed by the Australian Wine Research Institute (AWRI) and indicates markedly different proportions of the major tannin components than grape seeds or skin. Table 8 provides a summary of results for *Leucaena* tannin content compared with grape seed and skin. Detailed analysis including condensed tannin content (g/kg DM) is shown in Table A3. The degree of trihydroxylation of tannins indicates their bioactivity and the high % of this class of tannins in *Leucaena* indicates that they are likely to bind actively with protein in the rumen and affect digestibility and activity of microorganisms.

Table 8. Qualitative description of tannin composition (ratio of subunits) and galloylation % for *Leucaena* samples compared with grape seed and skin.

	mDP ¹	Cis/Trans ²	%Tri-OH ³	%Gall
Grape seed	Low	low	low	high
Grape skin	High	high	high	low
<i>Leucaena</i>	Low	low	high	low

¹mean degree of polymerisation [length of chain (compounds 1-3 v. 4-7)]; ²catechin subunits/epicatechin subunits; [stereochemistry (compounds 1,4 v. 2,3,5-7)]; ³extent of hydroxylation as % containing tri-hydroxylated subunits

In-vitro studies of anti-methanogenic effects of *Leucaena* investigated in BCCH6530 'The mechanism of antimethanogenic bioactivity of plants in the rumen' showed these tannins are likely to be involved in the anti-methanogenic effect of *Leucaena*.

Table A3. Mean concentration (g/kg DM), composition of tannins and galloylation % for Leucaena browse collected from Belmont Research Station and Brian Pastures.

Material	Site	year	Tannin (g/kg DM)			Tannin composition		
			PA	PA+LEM	mDP	cis/trans	%Tri-OH	%Gall
Leaf/stem	BRS	2014 ¹	27.83	58.38	4.26	7.9	57.6	5.9
Leaf/stem	BRS	2013 ²	69.56	118.09	4.38	6.79	39.5	6.2
Leaf/stem	BRS	2013 ²	64.05	112.26	4.17	6.11	40.8	5.1
Seed pod	BP	2013 ³	9.07	13.83	13.65	31.29	49.8	3.3

PA, proanthocyanidins (condensed tannins); LEM, late eluting material; mDP, mean degree of polymerisation [length of chain (compounds 1-3 v. 4-7)]; cis/trans, catechin subunits/epicatechin subunits; [stereochemistry (compounds 1,4 v. 2,3,5-7)]; %Tri-OH, hydroxylation as % containing tri-hydroxylated subunits; BRS, Belmont Research Station; BP, Brian Pastures; ¹March, ²June, ³October

1.3 Herd scale methane emissions

1.3.1 Belmont Research Station

Herd scale methane data obtained over four events during 2013/14 indicated that steers grazing Leucaena were generally found to have lower daily emissions (g/head) than those grazing Rhodes grass pastures only (Fig 3). Improved filtering criteria removed inconsistencies in data associated with wind conditions, path length and sensor differences. Uncertainties also exist in the bLS modelling and are not reported.

The in-situ cross-calibration procedure used to determine herd scale emission values for Belmont worked well except for the following.

For the Leucaena-March2013 measurements, path 2 cross-calibration was unstable (the multiplier was highly variable over the calibration period). We have no explanation for this behaviour, and chose not to use the path 2 laser line.

For the Pasture-June2013 paddock measurements, path 1 cross-calibration was similarly unstable. We chose not to use the path 1 laser line.

For both the Leucaena-March2014 and the Pasture-March2014 measurements there were no wind directions that allowed the cross-calibration of the path 2 laser line, and the path 2 laser line was not used.

Application of the revised filtering criteria resulted in similar trends between Leucaena and Rhodes grass grazing groups with relative differences of a similar magnitude. Nevertheless, results presented here are not necessarily absolute and should be regarded as indicative of herd scale emissions only. Data filtering significantly reduced the number of 10 min observation periods available for emission calculations. The amount of good emission data ranged from 7 to 292 periods depending on the campaign and the touchdown coverage criterion and ranged from 133 to 281

Across 2013 direct measures of herd scale emissions conducted in March (late wet season) and June (mid dry season) indicated that daily methane production (g/head) for steers grazing Leucaena was 18% less than those grazing Rhodes grass pastures. However, direct measures conducted in March 2014 indicated that steers grazing Leucaena had similar mean daily emissions compared with those grazing Rhodes grass (281 v. 279 g/head, respectively). However, the March 2014 comparison used touchdown coverage ≥ 0.95 to ensure the number of observations > 30 .

When daily emissions are extrapolated to an intensity basis using mean carcass weight, values for Leucaena finished steers were estimated to be 335 g CH₄/kg HDCW, compared with 596 g CH₄/kg HDCW for steers finished on Rhodes grass pastures only.

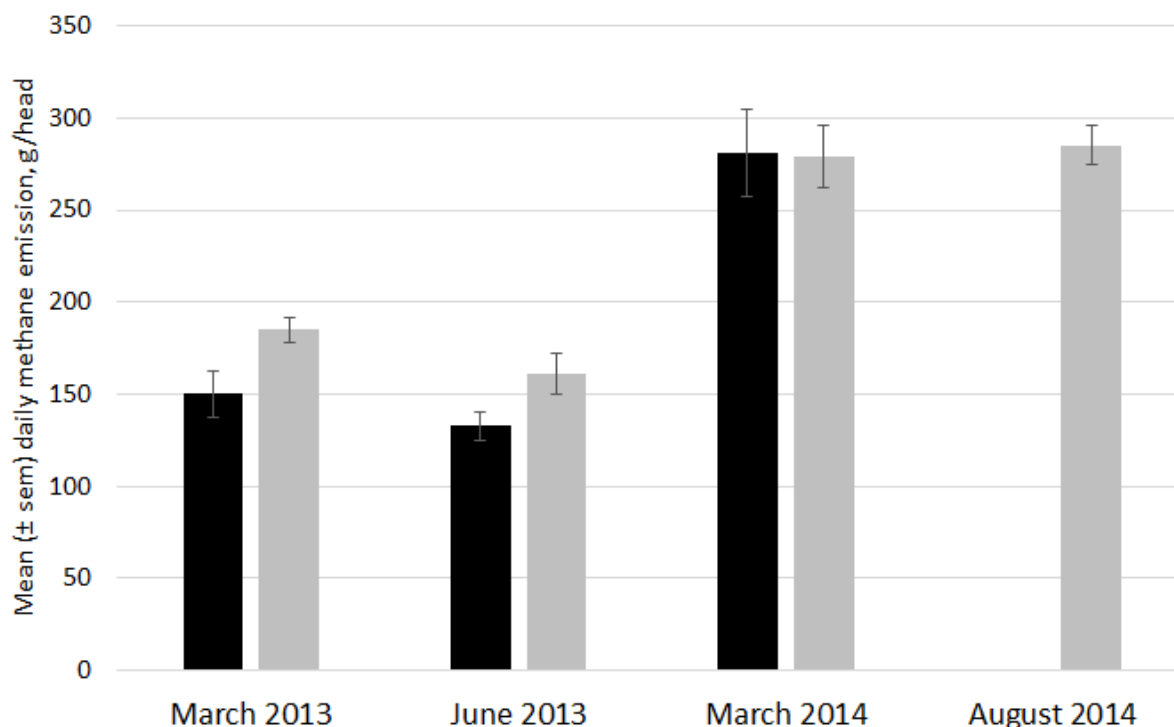


Fig 3 Mean (\pm sem) daily methane emissions for four independent events where steers grazed Leucaena (■) or Rhodes grass (■) pastures on Belmont Research Station, 2013-14. By August 2014 Leucaena steers had reached slaughter weight and exited the project.

No direct comparisons in emission data could be made in August 2014 as Leucaena finished steers ($n=14$) were slaughtered in June. The remaining number of animals was not sufficient to determine herd scale emissions and were subsequently managed on mixed grass pastures across Belmont.

1.3.2 Brian Pastures Research Station

Herd scale methane emission data obtained from steers grazing either leucaena or native grass pastures on Brian Pastures in September 2013 indicated similar trends to those on Belmont. Mean (\pm sem) LW for steers grazing Leucaena or native grass pastures during the measurement period was 241 ± 3.6 kg and 281 ± 3.8 kg, respectively. These cattle grazed Leucaena or unimproved grass pastures during the late dry season in 2013 when available forage had declined in nutritive value, intakes were limited and mean LWG was less than 0.3 kg/d based on 14 d intervals.

Mean (\pm sem) daily emission values after filtering the WindTrax output file for values outside of those described, were estimated to be 95 ± 7.9 g/head compared with 132 ± 7.1 g/head for steers grazing either Leucaena or native pasture, respectively. These results, although based on only one measurement period, 80 to 100 d after the commencement of the two grazing periods indicated that steers grazing Leucaena produced approximately 28 % less enteric methane than those grazing a native grass pasture dominated by *Dicanthium* sp.

ACTIVITY 2

Determine the improvements in productivity of cattle browsing leucaena plantations in north Queensland with grass pasture fed animals

2.1 Rumen fermentation

Mean total VFA concentration for Leucaena fed animals was not significantly different from cattle grazing grass pastures irrespective of the location of the grazing system and time of year (Figure 4).

The proportion of total VFA as propionate and butyrate as well as propionate:acetate ratio were significantly higher ($P < 0.05$) in all animals grazing Leucaena at Belmont Research Station on three different occasions over a two year period. In addition, branched chain fatty acids (iso-butyrate and iso-valerate) and longer chain fatty acid, caproate were also significantly higher ($P < 0.05$) for the Belmont Leucaena fed animals.

At Brian Pastures Research Station rumen fluid was collected on one occasion only. The Leucaena fed cattle had higher proportions of butyrate, branched chain fatty acids, valerate and caproate ($P < 0.05$) and propionate: acetate ratio tended to be lower.

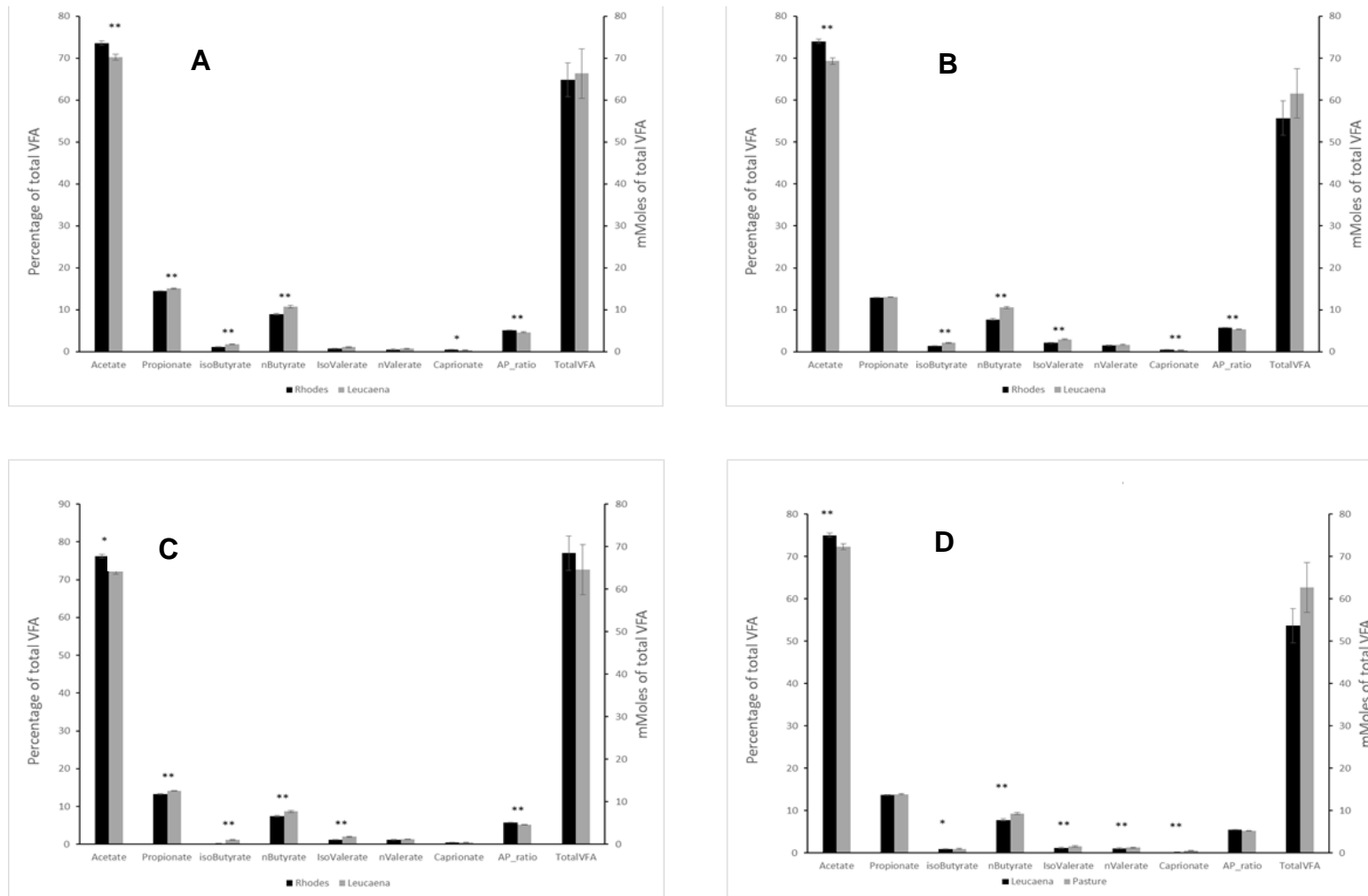


Figure 4. Total VFA concentration and molar proportions of C2-C6 VFA in the rumen of cattle grazing leucaena plantations compared with grass based pastures. A) Belmont March 2013; B) Belmont June 2013; C) Belmont March 2014 and D) Brian Pastures October 2013. Significance indicated at * $P < 0.05$ or ** $P < 0.001$

2.2 Animal productivity

Grazing of Leucaena and Rhodes grass pastures by two groups of cattle commenced on Belmont Research Station in January 2013. Individual liveweight data were recorded at regular intervals since 17 January 2013 (Figure 5). Mean daily gains, determined by linear regression, were 0.59 and 0.42 kg/d for Leucaena and Rhodes grass fed steers, respectively, over 600 d. At slaughter Leucaena steers were approximately 186 kg (LW) heavier than those finished on a Rhodes grass only pasture. Grazing periods to achieve slaughter weight, 400 d post weaning, were 500 d and 608 d for Leucaena and Rhodes grass groups, respectively. Carcass weights also differed by 100 kg and corresponding dressing percentages were similar at 49%. Fat depth was greater (by almost four fold) for Leucaena finished steers compared with Rhodes grass steers (Table 9). Carcass characteristics and MSA scores were recorded as described for all animals (Table 9). On assessment only three Rhodes grass finished steers qualified for MSA grading.

Grazing of Leucaena and native grass pastures by two groups of cattle commenced on Brian Pastures in July 2013. Individual liveweight data were recorded since initial allocation to grazing groups (Figure 6). Mean daily gains, determined by linear regression, were 0.58 and 0.28 kg/d for steers grazing Leucaena or grass pastures, respectively, over a total of 192 d. When the project was terminated at this site and the property destocked steers grazing Leucaena were 67 kg heavier than their counterparts off native grass pastures. No animals were available for slaughter or the determination of carcass characteristics or MSA scores.

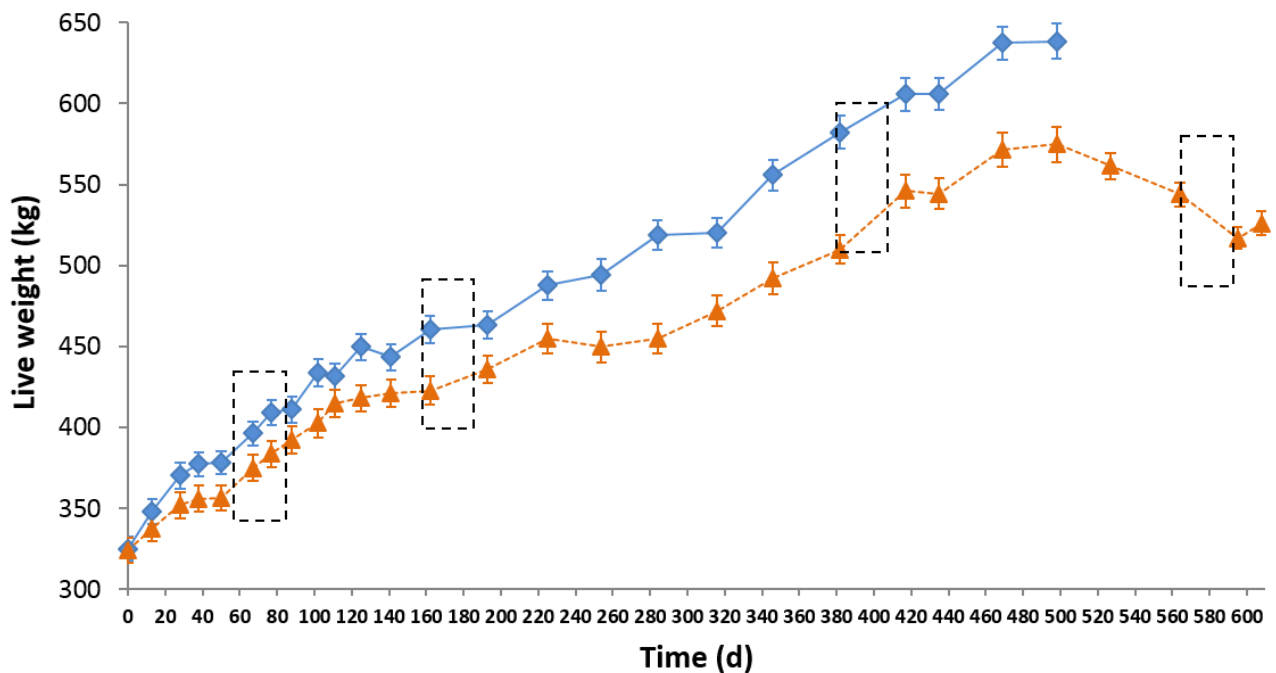


Figure 5. Mean (\pm sem) live weight (kg) collected from steers on Belmont Research Station grazing either Leucaena (\blacklozenge) or Rhodes grass (\blacktriangle) dominated pastures. Animals entered trial paddocks 17/01/2013, day 0. Each dashed rectangle indicates a methane measurement period; Mar/Apr 2013, Jun/Jul 2013, Mar/Apr 2014 and Aug 2014. Leucaena steers slaughtered June 2014. Rhodes grass steers slaughtered September 2014.

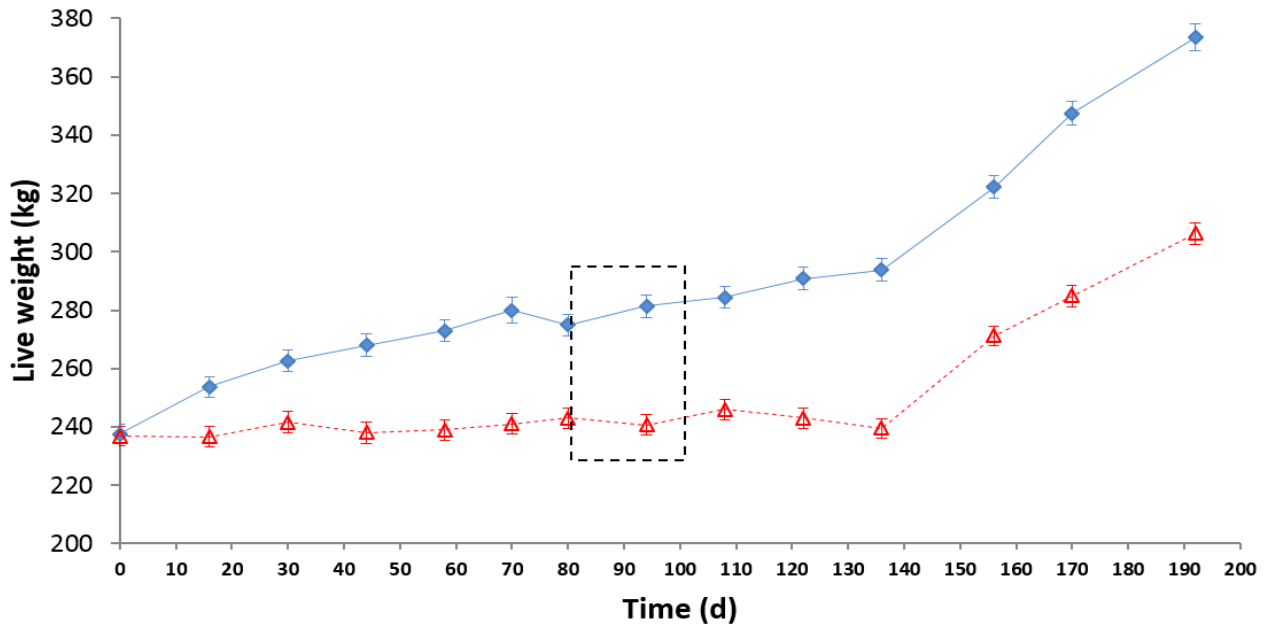


Figure 6. Mean (\pm sem) live weight (kg) of steers on Brian Pastures grazing either Leucaena (\blacklozenge) or native grass (\blacktriangle) pastures. Animals entered trial paddocks 08/07/2013, day 0. Dashed rectangle indicates the only methane measurement period; Sept/Oct 2013.

Table 9. Mean (\pm sem) carcass characteristics for steers finished with or without Leucaena; 500 d on Leucaena based pastures and 608 d on Rhodes grass dominated pastures, post weaning, on Belmont Research Station

<u>Carcass characteristic</u>	<u>Leucaena</u>	<u>Rhodes grass</u>
N	14	14*
Liveweight (kg)	687 \pm 10.6	501 \pm 8.5
Hot carcass weight (kg)	342 \pm 5.5	248 \pm 5.2
Dressing percentage	49.8 \pm 0.38	49.4 \pm 0.52
P8 fat depth (mm)	16.3 \pm 1.39	4.0 \pm 0.42
AUS-MEAT marbling score	0.8 \pm 0.15	0.3 \pm 0.33
MSA marbling score	330.0 \pm 17.67	207 \pm 12.01
Fat colour (subcutaneous)	2.1 \pm 0.20	1.3 \pm 0.33
Meat colour	2.5 \pm 0.23	3.0
Eye muscle area (cm ²)	75.2 \pm 2.94	71.7 \pm 0.33
Ossification	138 \pm 3.0	140
pHu	5.6 \pm 0.02	5.6 \pm 0.04

Fat colour determined using AUS-MEAT standard reference chips (AUS-MEAT 1996); *n=3 only for MSA grading purposes

ACTIVITY 3

Model the yearly reductions in methane based on unit of beef produced and carbon equivalent offsets associated with growing leucaena plantations

The effects of Leucaena on GHG emissions, production and profitability at the whole farm level for a property in northern Australia was modelled as described by Harrison *et al.*, (2015). Relative differences in methane emissions of up to 21 % was used to inform the modelling analyses based on estimated methane emissions for each measurement period conducted on Belmont Research Station from cattle on either Rhodes grass or Leucaena.

3.1. Scenario 1: Leucaena enterprises with equivalent stocking rates to the baseline. Allowing livestock to graze Leucaena increased animal growth rates and liveweight such that the number of cattle carried on farm was reduced in order to maintain stocking rate (Table 10). Even though this reduced total annual animal sales by 5%, total liveweight production and profitability increased by 8% and 17%, respectively. When carbon was valued at \$23 t/CO₂-e potential carbon offset income from farmer participation in a scheme such as the CFI contributed ~\$2900 to an additional ~\$13,000 gross margin received from a Leucaena enterprise run at the same stocking rate as the Rhodes grass enterprise (scenario 1). The higher crude protein content of Leucaena increased nitrous oxide production by 49% relative to Rhodes grass in scenario 1, but this did not substantially offset a 17% reduction in net emissions because nitrous oxide constituted a much smaller proportion of whole farm emissions than did methane (~5% and ~95%, respectively). In both cases, nitrous oxide from dung and urine was around double that from indirect sources including nitrogen lost as ammonia volatilisation.

3.2. Scenario 2: Leucaena enterprises with equivalent liveweight production to the baseline.

When annual liveweight production of the Leucaena enterprise was matched with that of the baseline, stocking rate and total livestock sales decreased by 8% and 12%, respectively (scenario 2). This resulted in a carbon offset (CFI) income of ~\$4000 at \$23 t/CO₂-e, though gross margin was around \$6000 less than Leucaena enterprises run at equivalent stocking rates (*cf.* scenarios 1 and 2). Scenario 2 had the lowest net farm emissions of all Leucaena scenarios examined, with reductions in total and methane emissions of more than 24%, although nitrous oxide emissions were still 38% greater than those of the Rhodes grass enterprise (Table 10).

3.3. Scenario 3: Leucaena enterprises with equivalent net farm emissions to the baseline

To match net farm emissions of the Leucaena enterprise with that of the baseline (scenario 3), stocking rate increased by 21%, resulting in a 14% increase in animal sales. Liveweight sales and gross margin increased even further (31% and 37% greater than the baseline, respectively), notwithstanding the fact that there was no carbon offset income (Table 10). In scenario 3 methane emissions were around 4% lower than baseline methane production and nitrous oxide emissions were around 81% greater, indicating the relative change in the emissions profile caused by introducing Leucaena when net farm emissions are maintained.

3.4 Average annual production, emissions and emissions intensity of each animal class

Steers aged 2–3 years and cows constituted the majority of emissions and liveweight carried on farm, with each accounting for 24–26% of liveweight carried and 22–24% of emissions from animals carried on farm. There was a tendency for Leucaena enterprises to shift total liveweight and emissions from cows to steers, but these effects were relatively small. The metric used to compute emissions intensity strongly influenced the relative differences between animal classes. Calves had the greatest emissions per unit liveweight, but the lowest emissions per animal. Bulls had the lowest emissions per unit liveweight and the highest emissions per animal. Irrespective of the metric used for computation, emissions intensities of all animal classes, except calves, were lower for enterprises that allowed livestock access to Leucaena.

Table 10. Annual production, gross margin and greenhouse gas (GHG) emissions of three alternative Leucaena enterprises in scenarios with equal numbers of adult equivalents (AE), equal total liveweight turnoff (LW) or equal net farm emissions to that of the baseline Rhodes grass enterprise. Carbon offset income from GHG emissions abatement was computed relative to the baseline assuming carbon prices of \$23 or \$10/t CO₂-e. Values in parentheses indicate percentage change relative to the baseline.

	Baseline	Scenario 1: leucaena equal AE	Scenario 2: leucaena equal LW	Scenario 3: leucaena equal emissions
Production				
Total adult equivalents (AE)	400	400 (0)	369 (-8)	483 (21)
Total cattle carried (head)	406	385 (-5)	355 (-12)	465 (15)
Total breeders mated (head)	205	194 (-5)	179 (-12)	235 (15)
Total cows and heifers sold (head)	71	67 (-5)	62 (-12)	81 (15)
Total steers and bullocks sold (head)	73	69 (-5)	64 (-12)	84 (15)
Total animals sold (head)	145	138 (-5)	127 (-12)	166 (14)
Total liveweight turnoff (t LW)	75.1	81.4 (8)	75.2 (0)	98.2 (31)
Economics(\$AUD)				
CFI income @ \$23/t CO ₂ -e (\$)	0	2,944	4,018	0
CFI income @ \$10/t CO ₂ -e (\$)	0	1,280	1,747	0
Gross margin with CFI @ \$23/t (\$)	79,647	93,021 (17)	86,959 (9)	109,166 (37)
Gross margin per adult equivalent (\$/AE)	199	233 (17)	236 (18)	226 (14)
Greenhouse gas emissions				
Total methane emissions (t CO ₂ -e/farm)	699	553 (-21)	511 (-27)	670 (-4)
Total nitrous oxide emissions (t CO ₂ -e/farm)	38	57 (49)	52 (38)	68 (81)
Net farm GHG emissions (t CO ₂ -e/farm)	738	610 (-17)	563 (-24)	738 (0)
Emissions per animal sold (t CO ₂ -e/head)	2.1	1.9 (-9)	1.9 (-9)	1.9 (-9)
Emissions per animal carried (t CO ₂ -e/head)	1.6	1.4 (-13)	1.4 (-13)	1.4 (-12)
Emissions per LW sold (t CO ₂ -e/t LW sold)	4.1	3.3 (-21)	3.3 (-21)	3.3 (-21)
Emissions per LW carried (t CO ₂ -e/t LW)	4.7	3.9 (-18)	3.9 (-18)	3.9 (-18)
Emissions intensity (t CO ₂ -e/t LW sold)	9.8	7.5 (-24)	7.5 (-24)	7.5 (-23)

3.5 Sensitivity analysis

The sensitivity of total liveweight production, emissions intensity and gross margin per adult equivalent (AE) was examined via $\pm 10\%$ variation in each of the main inputs. A preliminary analysis revealed that the sensitivity of most outputs of the baseline and Leucaena scenarios were similar. Total liveweight produced and emissions intensity were relatively insensitive to perturbation of measured variables (<3% variation). Gross margin per AE was more sensitive to reductions rather than increases in liveweight gain, suggesting diminishing returns per AE as liveweight gain increased. Gross margin per AE was most sensitive to prices received and was more than twice as sensitive to prices as to costs. Prices were attributed per unit liveweight whereas costs were mostly attributed per unit animal.

ACTIVITY 4

Elucidate the underlying rumen microbial structure and function that characterises the low methane phenotype of Leucaena fed cattle

4.1 Rumen metabolome analyses

In Leucaena fed animals, pyro glutamate and the amino acids valine, isoleucine and glutamic acid were significantly increased ($P < 0.05$) compared with grass fed animals (Table 11). The increased amino acid supply was also reflected in a significant increase in the amino acid metabolites 2-aminobutyric acid, 5-aminovaleric acid and 2-hydroxyglutaric acid.

Table 11. Significant fold changes in the rumen metabolome; amino acids and amines, organic acids, sugars and sugar phosphates, uracil, hypoxanthine and inosine for cattle grazing Leucaena compared with Rhodes grass pasture alone in July and August at Belmont Research Station.

	Belmont (June 2013)		Belmont (August 2014)	
	Rhodes grass	Leucaena + Rhodes grass	Rhodes grass + Rhodes grass	Leucaena
Amino Acids & Amines				
Valine	1.0	14.6 ± 0.3	1.0	7.1 ± 0.3
Isoleucine	1.0	19.9 ± 0.3	1.0	7.5 ± 0.4
Pyroglutamate	1.0	6.0 ± 0.2	1.0	4.1 ± 0.2
Glutamic acid	1.0	11.3 ± 0.3	1.0	10.2 ± 0.4
Organic acids				
Lactate	1.0	2.6 ± 0.3	1.0	1.4 ± 0.1
Glycolic acid	1.0	1.6 ± 0.1	1.0	1.1 ± 0.1*
Oxalate	1.0	2.7 ± 0.2	1.0	1.6 ± 0.3*
2-amino Butyric acid	1.0	6.4 ± 0.3	1.0	3.6 ± 0.3
Nicotinic acid	1.0	2.8 ± 0.1	1.0	2.5 ± 0.2
Succinate	1.0	2.6 ± 0.1	1.0	1.9 ± 0.1
Glycerate	1.0	2.4 ± 0.2	1.0	1.3 ± 0.2*
Glutaric acid	1.0	1.3 ± 0.1	1.0	1.4 ± 0.1*
Malate	1.0	3.1 ± 0.3	1.0	1.7 ± 0.1
Erythronate	1.0	1.4 ± 0.1	1.0	1.1 ± 0.1*
2-Hydroxyglutaric acid	1.0	2.9 ± 0.1	1.0	2.2 ± 0.1
5-aminovaleric acid	1.0	16.2 ± 0.3	1.0	6.9 ± 0.3
Sugars & Sugar Phosphate				
Glycerol-3-phosphate	1.0	3.0 ± 0.2	1.0	8.6 ± 0.6*
Myoinositol	1.0	3.6 ± 0.1	1.0	1.9 ± 0.1
Glucose-6-Phosphate	1.0	1.8 ± 0.1	1.0	1.0 ± 0.1*
Lactose	1.0	3.4 ± 0.3	1.0	1.4 ± 0.3*
Maltose	1.0	2.2 ± 0.2	1.0	1.0 ± 0.2*
Other				
Uracil	1.0	7.7 ± 0.2	1.0	3.9 ± 0.2
Hypoxanthine	1.0	16.1 ± 0.2	1.0	5.1 ± 0.2
Inosine	1.0	18.6 ± 0.2	1.0	4.1 ± 0.2

* Not significantly different from Rhodes grass control otherwise values are significantly different ($P < 0.05$)

Significant increases in lactose, malate and succinate were indicative of enhanced propionate production with Leucaena. The sugars, lactose and maltose were increased in Leucaena fed animals as were the intermediates of sugar metabolism, glucose-6-phosphate and glycerol-3-phosphate. In association with an increased supply of amino acids and sugars there was an increase in the nucleobases of RNA - uracil, inosine and hydroxyl xanthine.

4.2 Microbial analyses using quantitative PCR

Primary fibrolytic bacteria (*R. albus*, *R. flavefaciens* and *F. succinogenes*) varied in abundance in cattle grazing Leucaena compared with animals grazing grass pastures only. These differences were not consistent between animals at Belmont and Brian Pastures Research Station (Figure 7).

At Belmont a similar pattern was observed in samples from both 2013 (June) and 2014 (July). Leucaena fed animals had relatively higher populations of *R. albus*, but significantly lower numbers of *F. succinogenes* ($P < 0.01$) while there was no difference for *R. flavefaciens* between animals at the two sites.

At Brian Pastures only *R. albus* was different ($P < 0.01$) and at lower numbers in animals grazing *Leucaena*.

The abundance of total protozoa was 3-5 fold higher ($P < 0.01$) in all *Leucaena* fed animals irrespective of the location of the grazing system and time of year (Figure 8).

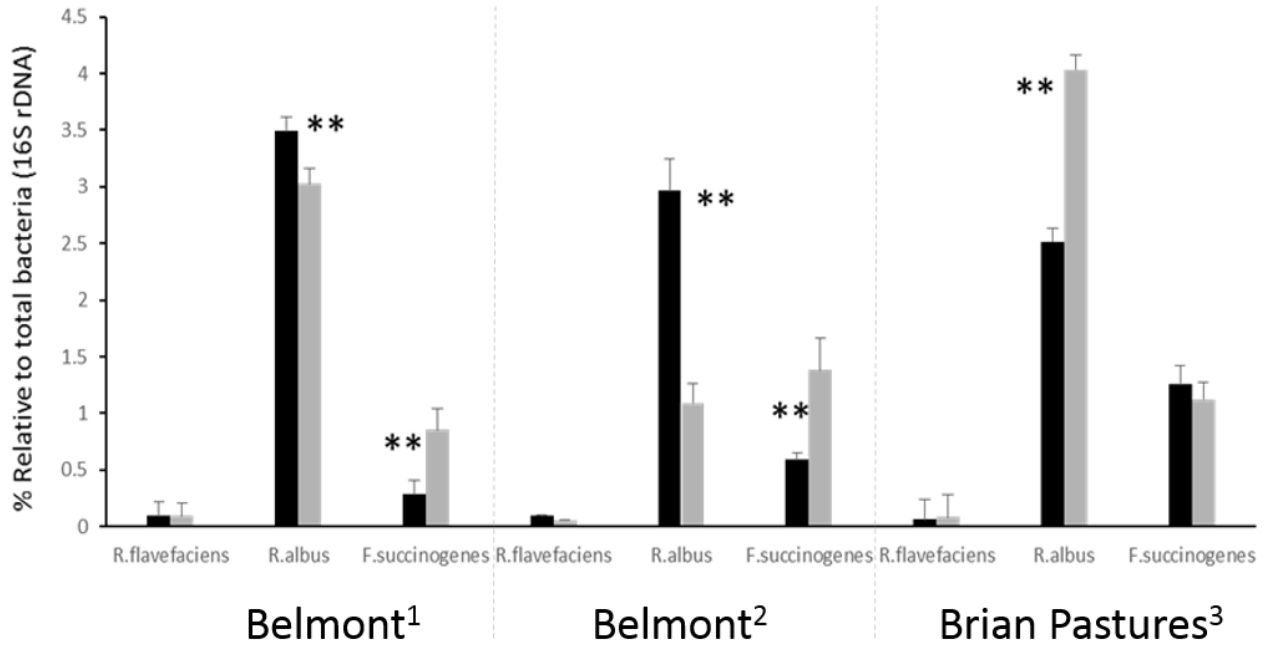


Figure 7. Relative abundance of primary fibrolytic bacteria in the rumen of cattle grazing *Leucaena* (■) or grass (■) based pastures at Belmont (¹June 2013; ²August 2014) and Brian Pastures (³October 2013) Research Stations. ** $P < 0.01$.

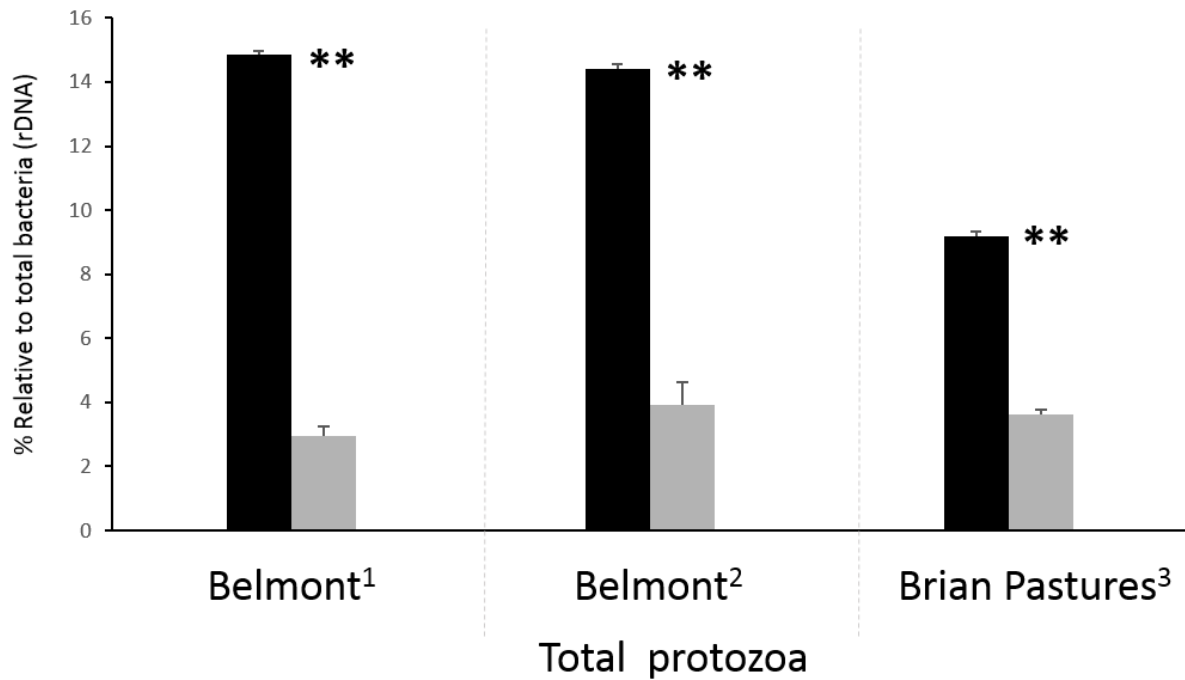


Figure 8. Relative abundance of total protozoa in the rumen of cattle grazing Leucaena (■) or grass (■) based pastures at Belmont (¹June 2013; ²August 2014) and Brian Pastures (³October 2013) Research Stations, ** $P < 0.01$.

4.3 Next generation sequence analysis

Sequence analysis of 16S rDNA from rumen microbial DNA of Leucaena and grass pasture fed cattle was used successfully in amplification strategies targeting the bacterial and archaeal populations of the rumen. At Belmont Research Station, total differences within the rumen population, due to diet, were evident and explained nearly 18% of the variance in the data when based on presence or absence of given bacterial species (Figure 9). Year and month of sampling had a smaller effect and represented less than 8% of the variance. Generally the rumen microbiomes share similar bacterial taxa, but show subtle and significant changes to the overall microbial populations between cattle feeding Leucaena compared with grass only. Phylum level analysis indicated that the same operational taxonomic units (OTUs) belonging to the Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia and Spirochaetes increased significantly ($P < 0.05$) in all animals fed Leucaena at both sites while other OTUs belonging to the Firmicutes, Bacteroidetes, Spirochaetes and Cyanobacteria decreased significantly relative to the grass pasture fed cattle (Figure 10, 11).

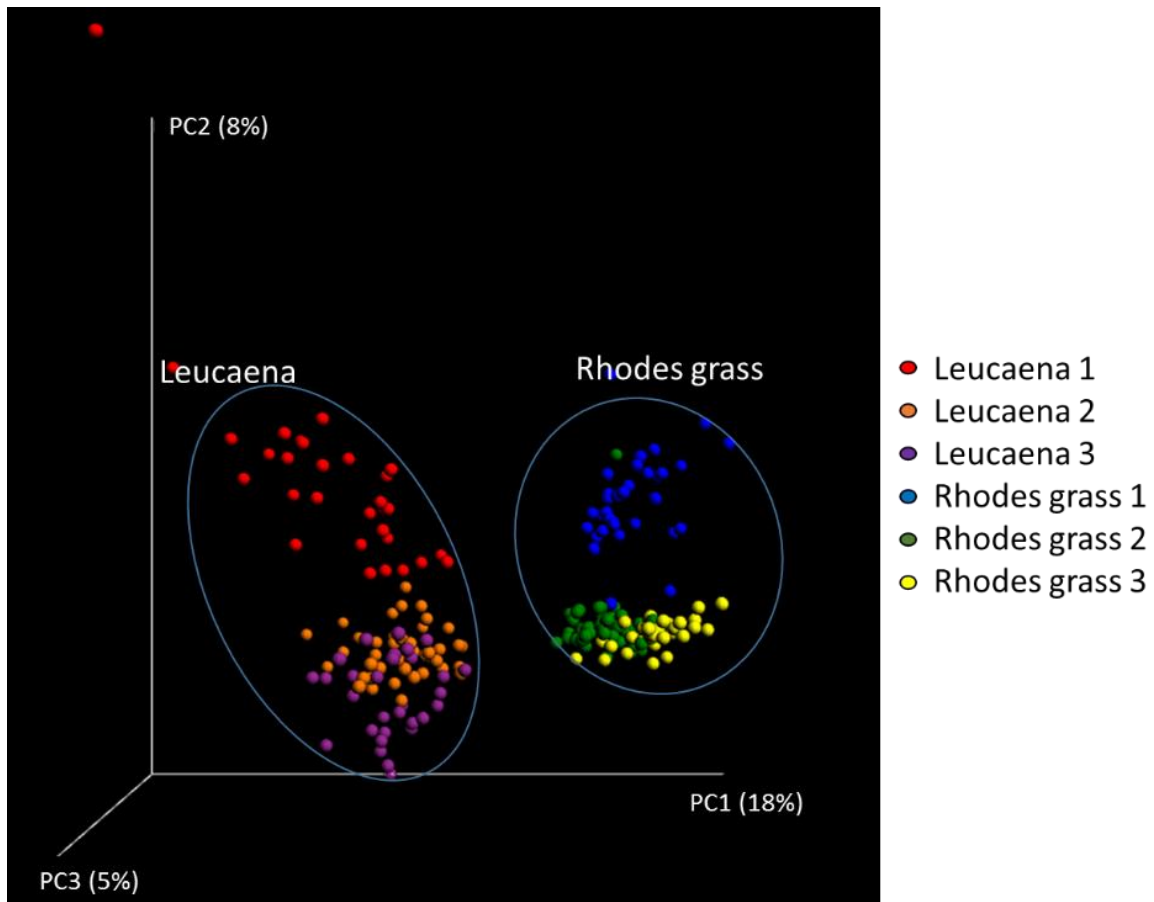


Figure 9.Principal component analysis (unweighted unifrac) for rumen bacterial composition analysis of individual animals on Belmont Research Station grazing Leucaena or Rhodes grass based pastures over two years; 1- March 2013; 2- June 2013; 3- July, 2014.

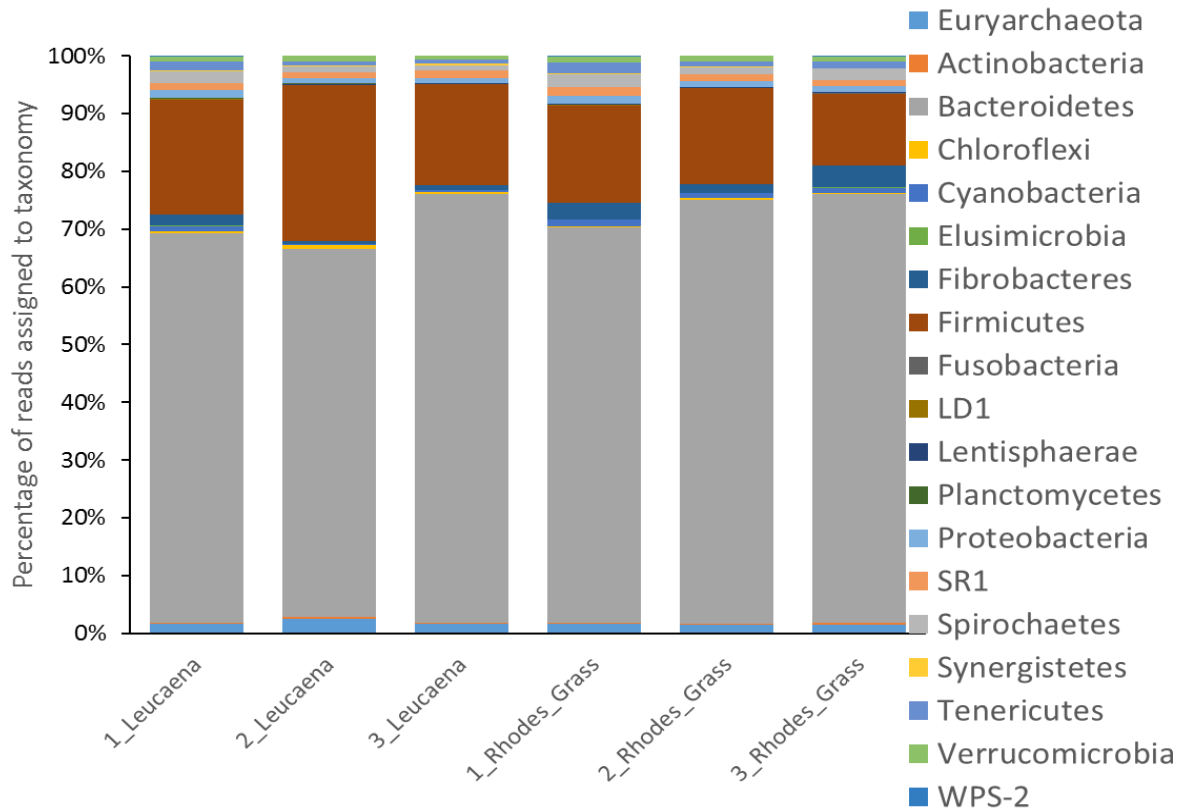


Figure 10. Distribution of taxonomic assignment to phylum level for rumen bacterial populations in cattle grazing Leucaena or Rhodes grass pastures at Belmont Research Station over two years; 1- March 2013; 2- June 2013; 3- July, 2014.

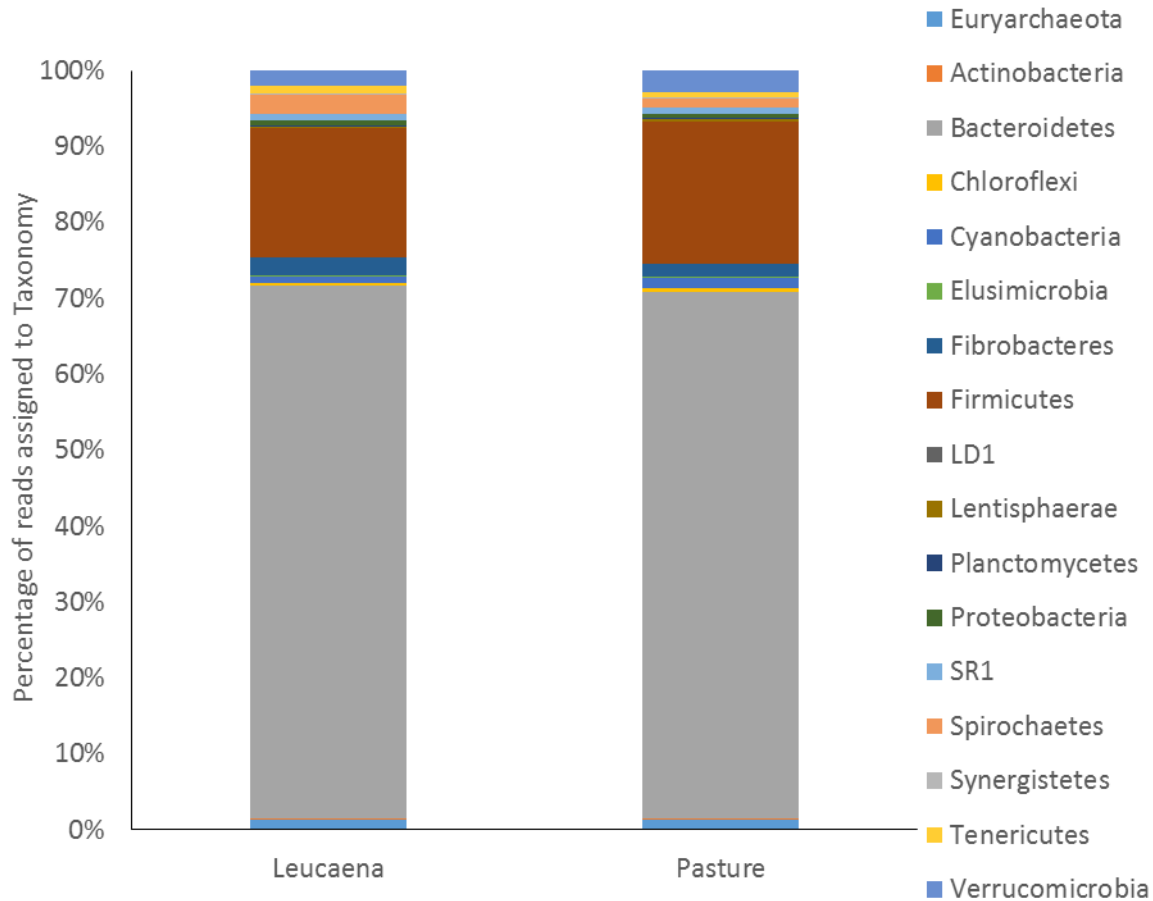
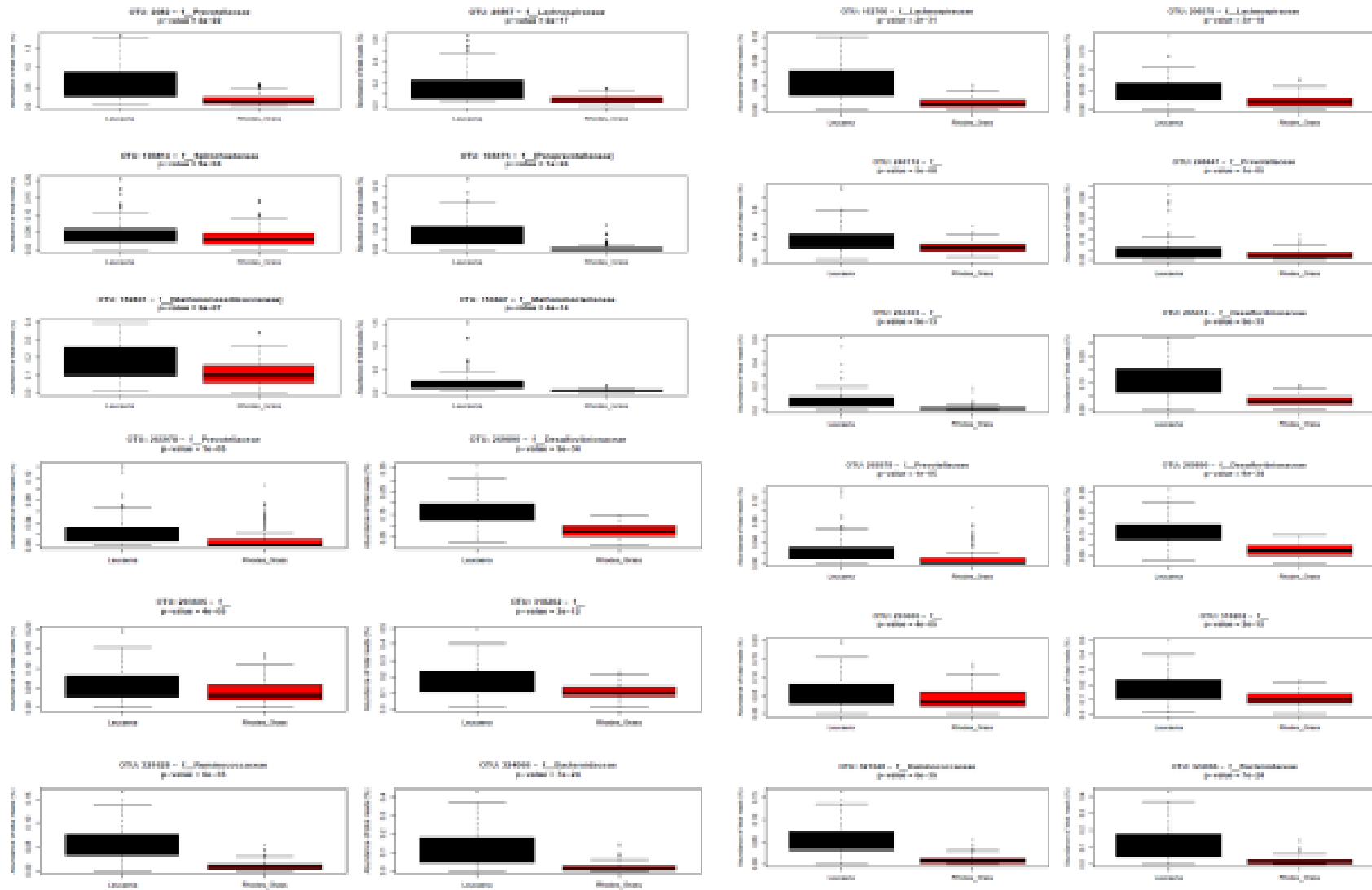


Figure 11. Distribution of taxonomic assignment to phylum level for rumen bacterial populations in cattle grazing Leucaena or naturalised grass pastures at Brian Pastures Research Station in October 2013.

Family level analysis indicated that the same OTUs belonging to the Lachnospiraceae, Prevotellaceae Spirochaetaceae, Desulfovibrionaceae, Ruminococcaceae, Bacteroidaceae, Veillonellaceae, and Spirochaetes increased significantly ($P < 0.05$) in all animals fed Leucaena at both Belmont and Brian Pastures Research Station. Other OTUs belonging to the Prevotellaceae, Erysipelotrichaceae and Bacteroidaceae decreased significantly relative to the pasture fed cattle (Figure 12, 13).

In addition, a single species closely related to *Synergistes jensei* was identified and found to be five to six times more abundant in those animals grazing Leucaena regardless of the location (Figure 14). Sequence similarity (96%) indicated that it was in the same genus, but would be classified as a new species. The ability of this species to degrade the toxin in Leucaena (dihydroxypyridine, DHP) is not known and its increase in abundance may only reflect the increased concentration of peptides from the digestion of Leucaena.



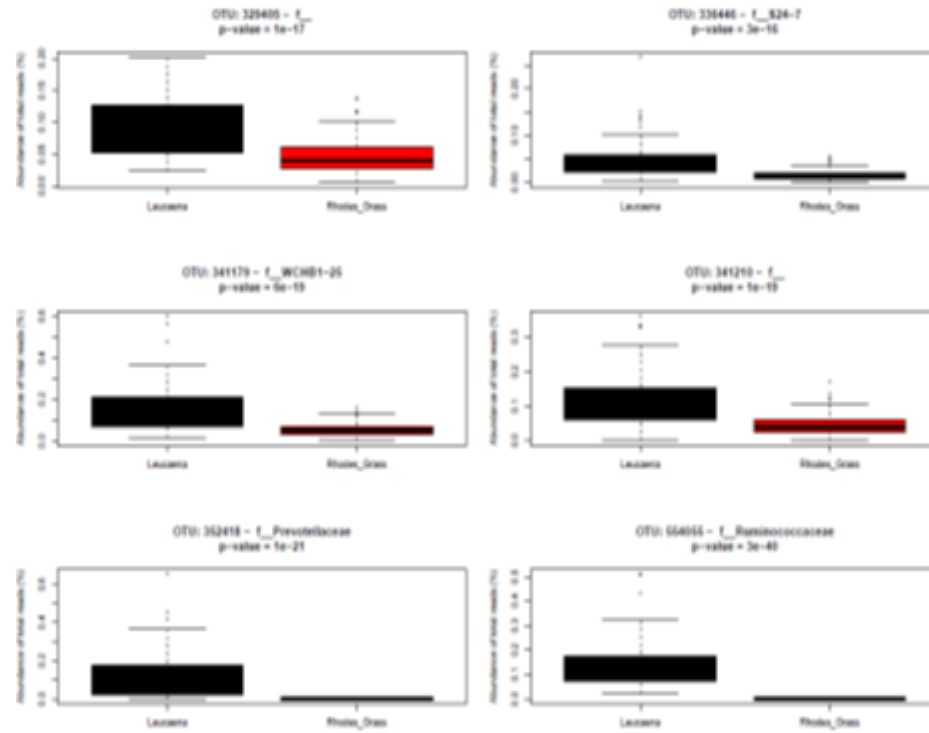


Figure 12. The relative abundance of operational taxonomic units at the family level which are significantly ($P < 0.05$) increased in cattle grazing Leucaena compared with Rhodes grass pastures at Belmont Research Station.



Figure 13. Relative abundance of operational taxonomic units at the family level which are significantly ($P < 0.05$) decreased in cattle grazing Leucaena compared with Rhodes grass pastures at Belmont Research Station.

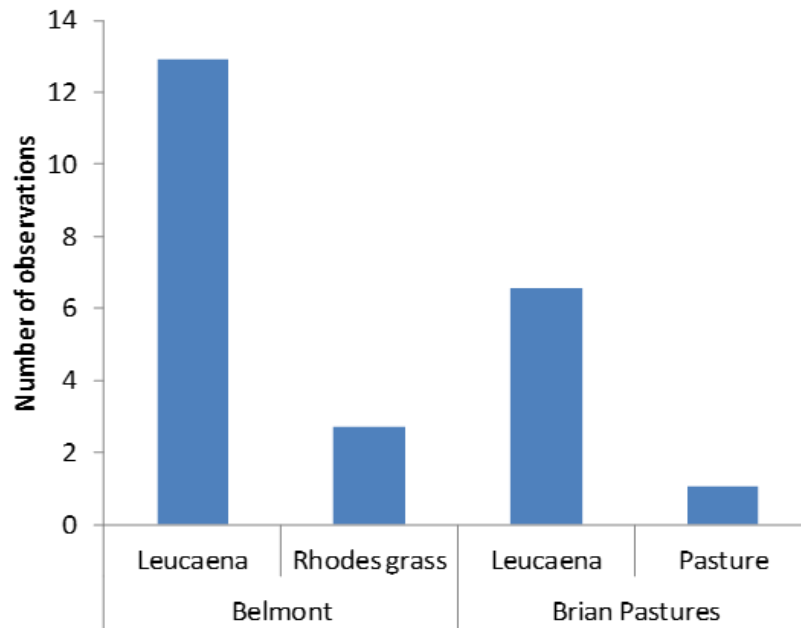


Figure 14. The relative abundance of a *Synergistes* spp. in cattle grazing Leucaena or grass based pastures on Belmont Research Station (July 2013) and Brain Pastures Research Station (October 2013).

Methanogen diversity was significantly different between cattle browsing Leucaena and those grazing Rhodes grass pasture at Belmont Research Station for all three sampling events (Figure 15). The differences were mainly due to an increase in methylotrophic *Methanosphaera* spp and unknown genera belonging to the *Methanobacteriaceae* family.

At Brian Pastures methanogen diversity changes associated with Leucaena in the diet were mainly related to an increase in species belonging to the *Methanosphaera* and Rumen Cluster C clade (*Methanoplamatales*). Interestingly the *Methanoplamatales* numbers declined significantly in the Brian Pastures animals on grass pasture which did not occur in the animals on grass pasture at Belmont Research Station (Figure 16).

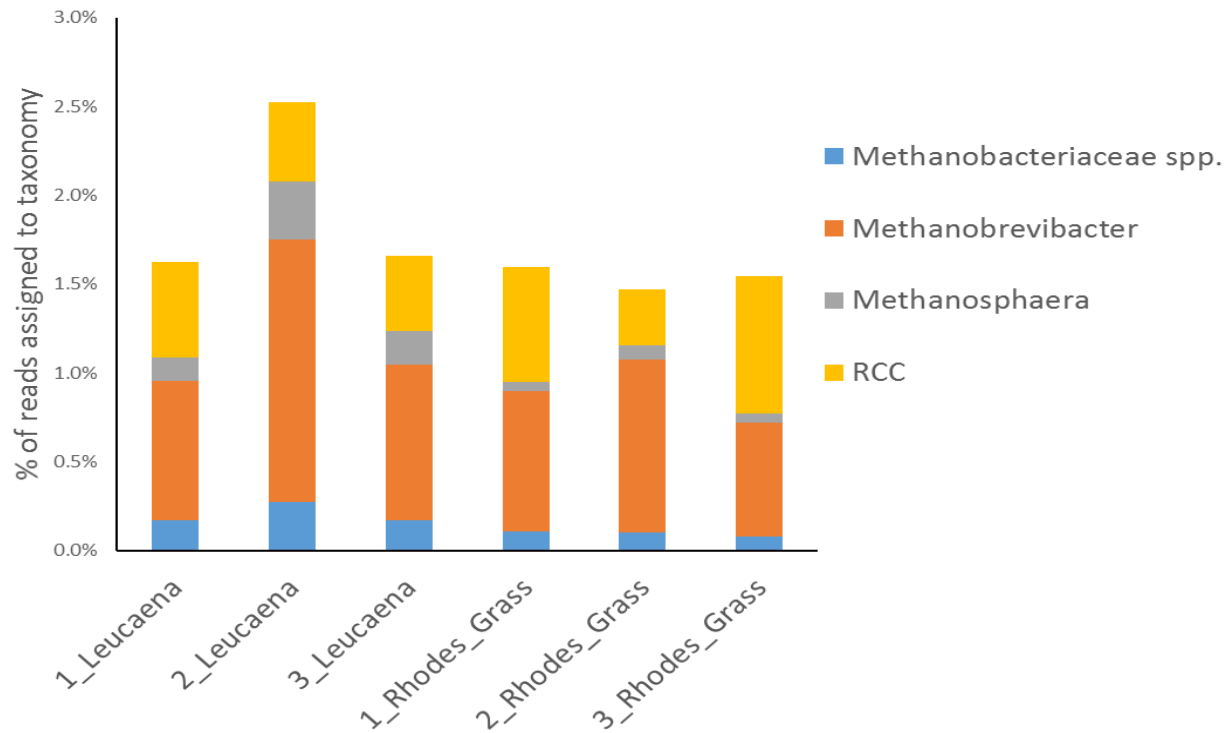


Figure 15. Distribution of taxonomic assignment to genus level for rumen methanogen populations (RCC, Rumen Cluster C) in cattle grazing Leucaena or Rhodes grass pastures at Belmont Research Station; 1- March 2013, 2- June 2013, 3 - July 2014.

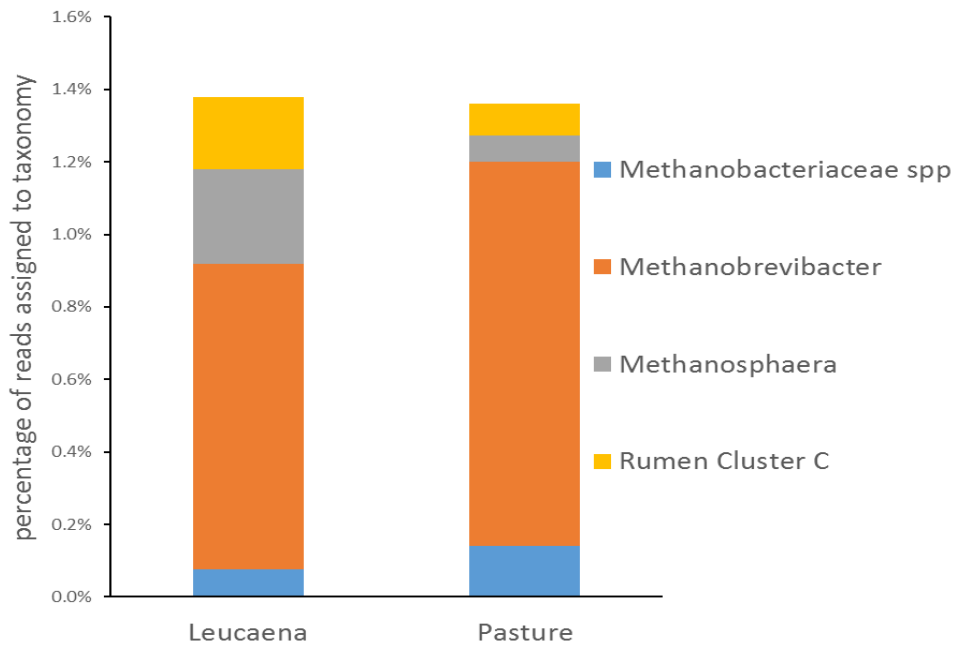


Figure 16 Distribution of taxonomic assignment to genus level for rumen methanogen populations in cattle grazing Leucaena or grass pastures at Brian Pastures Research Station; October 2013.

4. Discussion

ACTIVITY 1

Determine the reductions in methane emissions of cattle browsing Leucaena plantations in north Queensland compared with grass pasture fed animals

This activity has demonstrated that cattle grazing Leucaena have higher liveweight gain and lower methane emission intensity than those grazing C4 tropical grass pastures. Cattle in northern Australia generally graze C4 tropical pastures and consequently have lower annual liveweight gain and reproductive performance compared with other pasture based systems in southern Australia, resulting in higher methane emission intensity (Charmley *et al.* 2008).

The area planted to Leucaena plantations across northern Australia, is expected to expand by 300,000 to 500,000 ha over the next 10 years, (Reynolds 2013) and this development will undoubtedly have more cattle finished on this perennial shrub. Greater inclusion of Leucaena in beef production systems will reduce emissions intensity significantly for the sector as cattle reach a slaughter weight at a younger age. Above abatement benefits, Harrison *et al.* (2015) have demonstrated that financial returns resulting from greater animal production and maintenance of net emissions under leucaena are much greater than returns generated by an abatement scheme for mitigating greenhouse gas emissions.

Herd scale methane emissions determined on four occasions throughout 2013/14 using open path lasers indicated that mean emissions (g/d) were lower for cattle grazing Leucaena based pastures compared with those grazing Rhodes grass pastures only. Estimates of diet quality using F.NIRS indicated that up to 40 % (or approximately 2.7 kg/d DM) of the diet could be leucaena for those animals with continuous access to the browse shrub. Kennedy and Charmley (2012) fed *B. indicus* steers a range of diets containing both tropical grasses and legumes. In particular, the diets containing Rhodes grass, with 22 or 44 % Leucaena resulted in less methane (g/d basis), 7-15 % lower than when single grasses; Buffel, Bisset or Rhodes grass, were fed *ad lib.* While F.NIRS is able to predict the non-grass proportions in the diet of cattle grazing tropical pastures (Coates and Dixon 2007) it is currently not able to distinguish between individual legumes in the diet. Nevertheless, the methane reductions observed in this activity, particularly March 2014, for a diet containing approximately 30 % Leucaena are similar to those reported by Kennedy and Charmley (2012) when Leucaena accounted for 44% of the diet. Similar mitigation values (relative differences) were used by Harrison *et al.* (2015) from partial data sets originating from this work. While these values are not absolute due to the nature of field measurements, modelling assumptions and criteria used to maintain data integrity they do indicate trends over time in methane mitigation as a result of Leucaena feeding. Kennedy and Charmley (2012) reported mean values from individual animal data measured using open-circuit respiration chambers. The open path laser technique measures herd scale emissions only and individual animal means are calculated arithmetically, not directly, on a temporal (extrapolated to 24 h) and herd size (n) basis.

Methane abatement associated with Leucaena for all measurement periods (March, June 2013 and March 2014) was found to range from 0 to 19 %. Data analysis using a more refined and site specific filtering criteria was able to demonstrate a mean abatement benefit associated with Leucaena finishing on two occasions in 2013 of 18.2%, although this was associated with mean live weights of less than 500 kg. . By March 2014, emission values were similar for steers grazing either the Leucaena or Rhodes grass pastures. Uncertainties also exist in the BLS modelling and are not reported. Increasing the *tdcovthres* from 0.95 to 1.0 acted to reduce the standard deviation of the emission calculations (effectively removed outliers), particularly for the Leucaena-March 2013 results. This argues for using a high *tdcovthres* if possible. However, we noted that a large number of observations were lost as the threshold increased from 0.95 to 1.0. The uncertainties of the individual emission observations were large, often above 50%. This high uncertainty is due to the relatively low concentration rise downwind of the paddocks (ΔC) compared to the estimated uncertainty in the laser measurement (0.057 ppm). Initial data processing generated a number of negative emission rates in the dataset, some of which could be related to unsuitable wind statistics. In the vast majority of cases the negative values were not different from zero (the uncertainty range of the measurement spans zero). In this activity the negative emission values were, on examination, inevitable given the relatively small concentration rises being

measured, and these values were mirrored by erroneously large emission rates with similarly high uncertainty. Even though the measurement uncertainty of individual 10 min emissions was large, the uncertainty in the overall average emission rate was surprisingly small based on the March 2013 data set. This may have been due to the fact that the addition of random errors in the summing process (to give averages) tends to cancel out uncertainties: for the March 2013 data set the average daily emission rate for steers grazing Rhodes grass pastures was 185 g/head, and the measurement uncertainty was only 11 g/head.

Application of the revised filtering criteria resulted in similar trends between Leucaena and Rhodes grass grazing groups with relative differences of a similar magnitude, compared with those previously reported. Similarly, emissions intensities remained higher for steers grazing Rhodes grass pastures to slaughter compared with steers finished on Leucaena which had heavier carcasses almost 100 d earlier (596 v. 335 g CH₄/kg HDCW, respectively). Results presented here are not necessarily absolute and should be regarded as indicative of herd scale emissions only.

The CP content of the Leucaena biomass in June 2014 was the lowest (241 g/kg DM) compared with biomass collected in either March 2013 or 2014 (377 and 300 g/kg DM, respectively). Intakes and DM digestibilities, estimated by F.NIRS, were consistently higher for cattle grazing Leucaena compared with those grazing Rhodes grass only. Leucaena fed animals would have experienced a greater supply of amino acids and soluble carbohydrates compared with animals grazing a C4 grass only, which resulted in an apparent increase in microbial protein synthesis and sink for metabolic hydrogen. Both ADF and NDF fractions of the diet were also consistently lower for Leucaena material. The Leucaena material used by Kennedy and Charmley (2012) also contained the lowest NDF and highest GE concentrations of any tropical forage used in that study.

The presence of lipid material and condensed tannins (Mtenga and Laswaii 1994; Soltan *et al.*, 2013) in Leucaena may have also contributed to the reductions observed in emissions when cattle continuously graze Leucaena. Across the two years of the project, data indicated that methane emissions from Leucaena finished cattle may have been reduced compared with those from pasture based systems depending on the time of the year: late wet season v. mid dry season conditions. However, reductions in enteric methane with Leucaena may not have occurred during the dry season. Furthermore, the extrapolated measurements of methane emissions per day based on only periods of 5-6 h made by open-path laser measurements should be regarded as qualitative in nature until additional quantitative data can be generated to identify actual 24 h measurements under grazing conditions and validated by respiration chamber studies of animals fed varying levels of Leucaena. Further refinements and interrogation of the data from the open-path laser measurements of methane production is ongoing to account for inconsistencies identified with laser operation and wind conditions.

These outcomes: improved animal productivity and emissions reductions could contribute to the development of an abatement methodology for the northern beef industry.

ACTIVITY 2

Determine the improvements in productivity of cattle browsing leucaena plantations in north Queensland with grass pasture fed animals

The benefits associated with the inclusion of Leucaena in animal production systems has been documented, especially for sub-tropical environments (Shelton and Brewbaker 1998, Shelton and Dalzell 2007). When planted in hedge rows with tropical grass interrows, it forms one of the most productive, profitable and sustainable improved pasture options for northern Australia (Dalzell *et al* 2012).

In this activity a marked overall difference in animal productivity became apparent between the grazing groups, especially for LWG: Leucaena steers demonstrated a 170 g/d advantage over grass finished steers over a period of almost 500 d. Steers finished on Leucaena on Belmont Research Station achieved a mean slaughter weight 186 kg greater than their grass fed counterparts. Similarly, days to

slaughter was 108 d longer for grass finished steers compared with those finished on Leucaena. This possibly reflects the higher availability of dietary CP for steers consuming Leucaena browse which ranged from 241 to 376 g/kg DM, compared to 43 – 73 g/kg DM for the Rhodes grass pastures. Fast growing animals generally reach a slaughter weight sooner than slower growing contemporaries and are therefore younger at slaughter, within a market category, and present carcasses yielding more tender beef (Perry and Thompson 2005). Of the 18 Leucaena finished steers processed in June 2014, 14 graded within MSA specifications. In contrast, only three of the 14 grass finished steers slaughtered in September of the same year could be graded under MSA specifications. Ultimate pH determined on carcasses of steers from both grazing groups indicated an acceptable combination of animal handling, slaughter and processing to achieve a moderate rate of muscle pH decline.

F.NIRS analysis of diet quality confirmed that steers with access to Leucaena were consuming material higher in dietary N, with a DMD % up to 64 % and containing up to 30 % non-grass, assumed to be Leucaena browse. Commercial production systems utilising Leucaena based pastures typically target a Leucaena intake of 35 -40 % in the diet to achieve a LWG of 1.0 kg/d (Dalzell *et al.*, 2006). Live weight data collected throughout this activity demonstrated a LWG of approximately 0.9 kg/d for the first 125 d, compared with 0.75 kg/d for steers continuously grazing Rhodes grass pastures. Although these steers entered the project approximately 8 months after weaning, the effect of diet on growth path and long-term weight gain in beef cattle has been well documented (Tomkins *et al.*, 2006). In this activity steers managed on Leucaena pastures achieved an overall LWG (500 d) of 0.6 kg/d which was similar to the value reported by Tomkins *et al.*, (2006) for steers experiencing slow growth immediately post weaning, but then then re alimented on good quality grass pastures that supported a LWG \geq 0.6 kg/d.

Improved levels of animal productivity can be related back to increased efficiency of feed utilization and favourable changes in rumen fermentation patterns (Schelling, 1984). In all Leucaena fed cattle there was a significant shift in fermentation to more reduced end products such as propionate, butyrate and branched-chain fatty acids. Consequently, with an increase in butyrate and propionate, a shift down of approximately 8% in A: P could be associated with cattle grazing Leucaena browse and the redirection of energy into animal productivity.

ACTIVITY 3

Model the yearly reductions in methane based on unit of beef produced and carbon equivalent offsets associated with growing leucaena plantations

This activity has demonstrated that the use of Leucaena plantations in a beef finishing system improved total liveweight sales by 8 % and 31 % (scenarios 1 and 3), and that Leucaena reduced net farm emissions by 17 % and 24 % (scenarios 1 and 2). Together these factors accounted for a 23% reduction in whole farm emissions intensity, indicating that Leucaena has clear benefits in terms of reducing whole farm carbon footprint.

Under scenarios of equal adult equivalents or liveweight production, Leucaena reduced whole farm emissions by more than 17 %. Emissions intensity for all animal classes, except calves, was lower for enterprises that allowed livestock to browse Leucaena. There were no differences between emissions intensity of calves because young animals were not allowed access to Leucaena until 16 months of age.

In all cases, enterprises with Leucaena were more profitable than those with grass-only pastures. The potential to increase stocking rates with Leucaena has been demonstrated by previous work, suggesting that Leucaena plantations can support grazing at significantly higher stocking rates than grass pastures (Shelton and Dalzell, 2007). In scenarios modelled in this activity the greatest profits were achieved by matching net farm emissions with those of the baseline by increasing stocking rates. Higher profit was elicited by improved diet quality and enhanced liveweight gains over that of the baseline. Since gross margins were computed using fixed costs and prices, and since prices were shown to be a relatively sensitive input, future economic studies should account for costs associated with planting Leucaena and the time required to recover for the initial capital investment. Given that these models used experimental data measured over only two years, a future iteration on this work

might be to use a dynamic model to simulate Leucaena production over several years. It is expected that the use of a dynamic model would reveal important insights into seasonal production due to variability in the weather, particularly since Leucaena persists through drought, but is intolerant of heavy frosts (Shelton and Brewbaker, 1998).

Enterprises with Leucaena that had equivalent stocking rates or total liveweight production (scenarios 1 and 2) to comparable grass enterprises had lower net farm emissions, resulting in a potential carbon offset income of ~\$2900 and ~\$4000, respectively (when Australian Carbon Credit Units were valued at \$23/t CO₂-e). Together with increased productivity these scenarios increased farm profitability by 9 – 18 %. Such financial gains were less than half the additional gross margin realised by maintaining baseline levels of farm emissions and increasing liveweight production on Leucaena pastures. These results imply that graziers using Leucaena in beef enterprises would receive higher financial returns by simply using Leucaena to increase animal productivity and forego any GHG abatement income. This is not surprising given that an average tonne of beef is worth 69 – 159 times more than a tonne of CO₂-e (\$1592/t LWT v. \$23/t CO₂-e or \$10/t CO₂-e; October 2014). Irrespective of whether enterprises incorporating Leucaena grazing had the same number of adult equivalents, total liveweight production or net farm emissions compared with enterprises with subtropical pasture grasses only, the high nutritive value of Leucaena browse and anti-methanogenic properties has been shown (modelled) to potentially reduce emissions intensity by at least 23 %. The relatively high crude protein content of Leucaena increased emissions of nitrous oxide, but since enteric methane emissions were around an order of magnitude greater, effects of increased nitrous oxide on net farm emissions were small.

This study has shown that financial returns from improved animal production and maintenance of net emissions under Leucaena are much greater than returns from mitigating emissions and maintaining current stocking rates or total liveweight production. If GHG abatement schemes are to become financially attractive to farmers, and align with other income from interventions to livestock farming systems (Harrison *et al.*, 2014a, 2014b; Ho *et al.*, 2014), then the value of Australian Carbon Credit Units (ACCU) will have to increase substantially.

ACTIVITY 4

Elucidate the underlying rumen microbial structure and function that characterises the low methane phenotype of leucaena fed cattle

Metabolomic and VFA analyses indicated that Leucaena fed animals had an increased supply of amino acids and soluble carbohydrates which resulted in an apparent increase in microbial protein synthesis and sink for metabolic hydrogen. There was a shift in fermentation from acetate to longer chain fatty acids. The increase in propionate production appeared to occur via an increase in the succinate pathway. While there were significant changes in abundance of fibrolytic bacteria between Leucaena and grass fed animals the differences were relatively small and probably reflect the amounts of digestible fibre in the grazing diet. The large increase in protozoa in cattle grazing Leucaena probably reflects the increased protein supply in the diet.

Illumina DNA sequencing showed a consistent difference in the diversity of methanogens in cattle foraging Leucaena browse compared with grass pastures alone in both irrigated and dryland systems, with an increase in relative abundance of Methanospaera species as a proportion of the total methanogen population. Family level analysis indicated that the same bacterial species belonging to the Lachnospiraceae, Prevotellaceae Spirochaetaceae, Desulfovibrionaceae, Ruminococcaceae, Bacteroidaceae, Veillonellaceae, and Spirochaetes increased significantly in all animals fed Leucaena at both properties while other species belonging to the Prevotellaceae, Erysipelotrichaceae and Bacteroidaceae decreased significantly relative to pasture fed cattle. A consistent difference in the diversity of methanogens occurred in cattle foraging on Leucaena/grass production systems compared with grass pastures alone in both irrigated and dryland systems. The relative abundance of Methanospaera species as a proportion of the total methanogen population was higher in Leucaena fed animals, but more remarkable was the observation that the same species of Methanospaera was increased in cattle at both Brian Pastures and Belmont Research Stations. This demonstrates that the

Methanosphaera were responding specifically to Leucaena in the diet and may be responsible for differences in methane emissions. In a recent New Zealand study, a combination of metagenomic and metatranscriptomic methods have now shown that total methanogen numbers were similar between “low” and “high” methane producing sheep, but there were differences in the relative abundances of the methylotrophic *Methanosphaera* spp. (increased in “low methane” sheep) and the hydrogenotrophic *Methanobrevibacter gottschalkii* clade (increased in “high methane” sheep). The abundance of transcripts encoding most functions coordinating the hydrogenotrophic pathway was also significantly increased in high methane producing sheep. The “high methane” emitting animals have been postulated to possess a longer retention of feed within the rumen as well as alterations in the bacterial “ruminotype” increasing the levels of ruminal hydrogen, with coordinate elevated expression of genes encoding the hydrogenotrophic pathway and greater methane yield (Janssen, 2010; Kittelmann *et al.*, 2014) . It seems intuitive to suggest that the increased relative abundance of methylotrophic methanogens like Methanosphaera in “low methane” animals may relate to their capacity for alcohol-fuelled methanogenesis when the bacterial ruminotype favours less hydrogen production during fermentation (Janssen and Kirs, 2008; Attwood *et al.*, 2011).

Increases in relative abundance of the same bacterial species belonging to the Families Lachnospiraceae, Prevotellaceae, Veillonellaceae and Ruminococcaceae occurred in cattle browsing leucaena on both Belmont and Brian Pastures Research Stations and at different times of the year which demonstrates a specific response to Leucaena in the diet. These species are characteristically involved in the fermentation of carbohydrate to propionate, longer chain fatty acids (> C2) and short chain alcohols (methanol and ethanol). It is likely, therefore, that the shift in bacterial populations and metabolism associated with Leucaena resulted in less metabolic hydrogen being produced for hydrogenotrophic methanogens due to microbial protein and longer chain fatty acids acting as sinks for hydrogen. An increase in short chain alcohols from the fermentation of pectin and xylan in Leucaena would acts as precursors for the methylotrophic methanogens Methanosphaera, which increased in abundance. We propose that these shifts in the bacterial and methanogen populations are the likely microbial basis for alterations in methanogenesis in Leucaena based cattle production systems. It was not possible to determine whether Leucaena tannins also played a role in this process but *in-vitro* evidence from the studies in BCCH6530 ‘The mechanism of antimethanogenic bioactivity of plants in the rumen’ suggest they contribute to a reduction in methane production.

5. Significance of findings for Australian agriculture

Collectively this project and other studies are demonstrating that Leucaena grazing enterprises are likely to reduce methane emissions compared with production systems based on native or naturalised grass pastures as well as enhancing sequestration of carbon in soil. Apart from the greenhouse gas abatement potential, Leucaena enterprises in northern Australia will provide a better feed-base for finishing cattle, and are likely to increase profitability through improvements in herd structure, liveweight gains and carcass quality. Existing practice in the cattle industry along with economic modelling a range of scenarios demonstrates that planting Leucaena into existing pastures is attractive and could be used to drive change in farming practice particularly in northern Australia.

6. Future research needs

Direct measurements for emission intensities

- methane emissions measured on Belmont Research Station were only determined on four occasions over 595 d. An increase in emission measurement frequency would provide more data that could be related to animal growth path, especially immediately pre weaning and across different grazing regimes. This work would generate better emission intensity values, especially if continued through to slaughter

- the comparison between irrigated and dryland Leucaena finishing systems remains unresolved. Further work may identify substantially different emission intensity values, but needs to be conducted at similar time points in the production system.

Dynamic Economic modelling

- economic studies should account for costs associated with planting Leucaena and the time required to recover for the initial capital investment. Given that these models used experimental data measured over only two years, a future iteration on this work might be to use a dynamic model to simulate Leucaena production over several years.
- the use of a dynamic model would reveal important insights into seasonal production due to variability in the weather, particularly since Leucaena persists through drought, but is intolerant of heavy frosts

Microbial diversity and rumen function

- methanogen diversity was found to be significantly different between cattle browsing Leucaena and Rhodes grass pastures. While differences have been related to an increase in *Methanosphaera* spp identifying the unknown methanogens belonging to the Methanobacteriaceae family would be advantageous in understanding the role of the rumen microbiome with changes in the feed base.
- the potential discovery of a new species of *Synergistes* which was found to be five to six times more abundant in those animals grazing Leucaena suggests more work is required to determine the actual role of *S. jonseii* in the degradation of mimosine and use in Leucaena based finishing systems

7. Publications and communications

Tomkins, N., Denman, S., McGavin, S., Padmanabha, J. and McSweeney, C (2014) *Leucaena (L. leucocephala)* based pastures have the potential to reduce methane emissions for beef cattle. *Proceedings Australian Society of Animal Production***30**, 284

Harrison, M., McSweeney, C., Tomkins, N. and Eckard, R (2015) Improving greenhouse gas emissions intensities of subtropical and tropical beef farming systems using *Leucaena leucocephala*. *Agricultural Systems***136**, 138-146. doi: 10.1016/j.agsy.2015.03.003

FutureBeef website. <http://futurebeef.com.au/resources/projects/can-leucaena-plantations-help-reduce-greenhouse-gas-emissions-from-cattle/>

Project fact sheet; [Impact of Leucaena feed systems on methane emissions and productivity in cattle.](#)

8. References

Allison MJ., Mayberry WR., McSweeney CS. and Stahl DA. (1992) *Synergistes jonsei*, gen. nov., sp. nov.: A rumen bacterium that degrades toxic pyridinediols. *System. Appl. Microbiol.* **15(4)**: 522-529. doi:10.1016/S0723-2020(11)80111-6

Attwood GT., Altermann E., Kelly WJ., Leahy SC., Zhang L. and Morrison M. (2011) Exploring rumen methanogen genomes to identify targets for methane mitigation strategies. *Anim. Feed Sci. Tech.* **166-167**: 65-75. doi:10.1016/j.anifeedsci.2011.04.004

AUS-MEAT. (1998) The AUS-MEAT chiller assessment system. *Aus-Meat Ltd.* Brisbane, QLD.

Caporaso JG., Kuczynski J., Stombaugh J., Bittinger K., Bushman FD., Costello EK., Fierer N., Peña AG., Goodrich JK., Gordon JL., Huttley GA., Kelley ST., Knights D., Koenig JE., Ley RE., Lozupone CA., McDonald D., Muegge BD., Pirrung M., Reeder J., Sevinsky JR., Turnbaugh PJ., Walters WA., Widmann J., Yatsunencko T., Zaneveld J. and Knight R. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods***7(5)**: 335-336. doi:10.1038/nmeth.f.303

Chalupa W. (1976) Degradation of amino acids by the mixed rumen microbial population. *J. Anim. Sci.***43(4)**: 828-834.

Charmley E. (2012) Mitigation of methane emissions from the northern beef herd. Using open path lasers to acquire direct measurements of methane emissions from cattle grazing northern pastures. Final Report (B.CCH.1004). *Meat & Livestock Aust. Ltd.* North Sydney, NSW, 2059.

Coates DB. and Dixon RM. (2007) Faecal near infrared reflectance spectroscopy (F.NIRS) measurements of non-grass proportions in the diet of cattle grazing tropical rangelands. *Rangeland J.***29(1)**: 51-63. doi:10.1071/RJ07011

Coates DB. and Dixon RM. (2008) Faecal near infrared reflectance spectroscopy estimates of diet quality and responses to nitrogen supplements by cattle grazing *Bothriochloa pertusa* pastures. *Aust. J. Exp. Agric.* **43(6-7)**: 829-834. doi:10.1071/EA08004

Dalzell S., Shelton M., Mullen B., Larsen P. and McLaughlin (2006) *Leucaena*. A guide to establishment and management. Meat & Livestock Australia Ltd. Sydney, Australia

- Dalzell S., Burnett D., Dowsett J., Forbes V. and Shelton M (2012) Prevalence of mimosine and DHP toxicity in cattle grazing *Leucaena leucocephala* pastures in Queensland, Australia. *Anim. Prod. Sci.***52**: 365-372
- Denman SE. and McSweeney CS. (2006) Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol. Ecol.***58(3)**: 572-582. doi:10.1111/j.1574-6941.2006.00190.x
- Denman SE., Tomkins NW. and McSweeney CS. (2007) Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiol. Ecol.***62(3)**: 313-322. doi:10.1111/j.1574-6941.2007.00394.x
- Dong Y., Bae HD., McAllister TA., Mathison GW. and Cheng KJ. (1997) Lipid induced depression of methane production and digestibility in the artificial rumen system (RUSITEC). *Can. J. Anim. Sci.***77(2)**: 269-278.
- Flesch TK., Wilson JD., Harper LA., Crenna BP., Sharpe RR. (2004) Deducing Ground-to-Air Emissions from Observed Trace Gas Concentrations: A Field Trial. *J. Appl. Met. Climat.* **43**: 487-502. doi.org/10.1175/1520-0450(2004)043<0487:DGEFOT>2.0.CO;2
- Flesch TK., Wilson JD., Harper LA., Todd RW. and Cole NA. (2007) Determining ammonia emissions from a cattle feedlot with an inverse dispersion technique. *Agric. Forest Met.***144**: 139-155. doi: 10.1016/j.agrformet.2007.02.006
- Flesch TK., McGinn SM., Chen D., Wilson JD. and Desjardins RL. (2013) Data filtering in bLS calculation of emissions rates: Improvements to increase data retention. *Adv. Anim. Biosci.***4**:472.
- Flesch, T.K., McGinn SM., Chen D., Wilson JD. and Desjardins RL. (2014) Data filtering for inverse dispersion calculations. *Agric. Forest Meteorol.* **198-199**: 1-6.
- Flesch TK., Wilson JD., Harper LA., Crenna BP. and Sharpe RR. (2004) Deducing ground-air emissions from observed trace gas concentrations: A field trial. *J. Appl. Meteorol.***43(3)**:487-502.
- Google, Digital Globe (2015). [Satellite image]. Retrieved March 29, 2015 from <http://www.earth.google.com>
- Harrison, MT., Christie, KM., Rawnsley, RP. and Eckard, RJ. (2014a) Modelling pasture management and livestock genotype interventions to improve whole-farm productivity and reduce greenhouse gas emissions intensities. *Anim. Prod. Sci.***54**: 2018–2028. <http://dx.doi.org/10.1071/AN14421>.
- Harrison MT., Jackson T., Cullen BR., Rawnsley RP., Ho, C. and Cummins L. (2014b) Increasing ewe genetic fecundity improves whole-farm production and reduces greenhouse gas emissions intensities: 1. Sheep production and emissions intensities. *Agric. Syst.***131**: 23–33. <http://doi:10.1016/j.agsy.2014.07.008>.
- Harrison MT., McSweeney CS., Tomkins NW and Eckard, RJ (2015) Improving greenhouse gas emissions intensities of subtropical and tropical beef farming systems using *Leucaena leucocephala*. *Agric. Sys.***136**: 138-146.
- Ho, C., Jackson, T., Harrison, M.T., Eckard, R.J., 2014. Increasing ewe genetic fecundity improves whole-farm production and reduces greenhouse gas emissions intensities: 2. Economic performance. *Anim. Prod. Sci.* **54**, 1248–1253. <http://dx.doi.org/10.1071/AN14309>

- Hunt LP. (2008) Safe pasture utilisation rates as a grazing management tool in extensively grazed tropical savannas of northern Australia. *Rangeland J.***30(3)**: 305-315. doi:10.1071/RJ07058
- Janssen PH. and Kirs M. (2008) Structure of the Archaeal Community of the Rumen. *Appl. Environ. Microb.* **74(12)**:3619-3625.doi:10.1128/AEM.02812-07
- Janssen PH. (2010) Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Anim. Feed Sci.Tech.***160(1-2)**:1-22. doi:10.1016/j.anifeedsci.2010.07.002
- Johnson KA. and Johnson DE. (1995) Methane emissions from cattle. *J. Anim. Sci.***73(8)**: 2483-2492.
- Kennedy PM. and Charmley E. (2012) Methane yields from Brahman cattle fed tropical grasses and legumes. *Anim. Prod. Sci.***52(4)**: 225-239. doi:10.1071/AN11103
- Kittelmann S., Pinares-Patiño CS., Seedorf H., Kirk MR., Ganesh S., McEwan JC. and Janssen PH. (2014) Two Different Bacterial Community Types Are Linked with the Low-Methane Emission Trait in Sheep. *PLoS ONE***9(7)**: e103171. doi:10.1371/journal.pone.0103171
- Koike S. and Kobayashi Y. (2001) Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. *FEMS Microbiol. Lett.***204(2)**: 361-366. doi:10.1016/S0378-1097(01)00428-1
- Loh Z., Chen D., Bai M., Naylor T., Griffith D., Hill J., Denmead T., McGinn S. and Edis R. (2008) Measurement of greenhouse gas emissions from Australian feedlot beef production using open-path spectroscopy and atmospheric dispersion modelling. *Aust. J. Exp. Agr.***48**: 244-247. doi:10.1071/EA07244
- McAllister TA., Okine EK., Mathison GW. and Cheng KJ. (1996) Dietary, environmental and microbiological aspects of methane production in ruminants. *Can. J. Anim. Sci.***76(2)**: 231-243.
- McGinn SM., Chen D., Loh Z., Hill J., Beauchemin KA. and Denmead OT. (2008) Methane emissions from feedlot cattle in Australia and Canada. *Aust. J. Exp. Agr.***48(1-2)**: 183-185.
- McGinn SM., Turner D., Tomkins N., Charmley E., Bishop-Hurley G. and Chen D. (2011) Methane emissions from grazing cattle using point-source dispersion. *J. Environ. Qual.***40**:22-27. doi:10.2134/jeq2010.0239
- McGinn SM., Flesch TK., Coates TW., Charmley E., Chen D., Bai M. and Bishop-Hurley G. (2015) Evaluating dispersion modelling options to estimate methane emissions from grazing beef cattle. *J. Environ. Qual.* **44**:97-102. doi:10.2134/jeq2014.06.0275
- McMurdie PJ. and Holmes S. (2013) phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS ONE.***8(4)**:e61217. doi:10.1371/journal.pone.0061217
- Moya D., Calsamiglia S., Ferret A., Blanch M., Fandiño JI., Castillejos L. and Yoon I. (2009) Effects of dietary changes and yeast culture (*Saccharomyces cerevisiae*) on rumen microbial fermentation of Holstein heifers. *J. Anim. Sci.***87(9)**: 2874-2881. doi:10.2527/jas.2008-1446
- Mtenga LA. and Laswai GD. (1994) *Leucaena leucocephala* as feed for rabbits and pigs: detailed chemical composition and level of inclusion on performance. *Forest Ecol. Manag.***64(2-3)**: 249-257.doi:[10.1016/0378-1127\(94\)90299-2](https://doi.org/10.1016/0378-1127(94)90299-2)
- Perry D. and Thompson J. (2005) The effect of growth rate during backgrounding and finishing on meat quality traits in beef cattle. *Meat Sci.* **69**: 691-702.

- Prins RA., Van Hal-Van Gestel JC. and Counotte GHM. (1979) Degradation of amino acids and peptides by mixed rumen microorganisms. *Zeitschrift für Tierphysiologische Tierernahrung U Futtermittelkunde* **42**: 333-339.
- Reynolds B. (2013) Ensuring that productive Leucaena can help the livestock industry. Final report (P.PSH.0490) *Meat & Livestock Aust. Ltd.* North Sydney, NSW, 2059.
- Roessner U., Luedemann A., Brust D., Fiehn O., Linke T., Willmitzer L. and Fernie AR. (2001) Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems. *Plant Cell***13(1)**: 11-29. doi:10.2307/3871150
- Schelling, GT. (1984). Modes of action in manipulating rumen function. *In Manipulation of Growth of Farm Animals* (ed. J F. Roche and DO. O'Callaghan), pp. 185-210. Kluwer Academic publishers Group.
- Shelton, H.M., Brewbaker, J.L., 1998. Leucaena leucocephala – the most widely used forage tree legume. In: Gutteridge, R.C., Shelton, H.M. (Eds.), *Forage Tree Legumes in Tropical Agriculture*. Department of Agriculture, The University of Queensland, Queensland, Australia. <<http://www.fao.org/ag/agp/AGPC/doc/Publicat/Gutt-shel/x5556e00.htm#Contents>>.
- Shelton, M., Dalzell, S., 2007. Production, economic and environmental benefits of leucaena pastures. *Trop. Grassl.* **41**, 174–190.
- Soltan, YA., Morsy AS., Sallam SM., Luca RC., Louvandini H., Kreuzer M. and Abdall AL. (2013) Contribution of condensed tannins and mimosine to the methane mitigation caused by feeding Leucaena leucocephala. *Arch. Anim. Nutr.* **67**: 169-184.
- Stevenson IL. (1979) The effect of L-alpha-amino-n-butyric acid on growth and production of extracellular isoleucine and valine by Eubacterium ruminantium and a related rumen isolate. *Can. J. Microbiol.***25(12)**: 1394-1400.
- Sultana H., Hussain-Yusuf H., Takahashi T., Morita T. and Onodera R. (2003) A quantitative study on arginine catabolism by mixed ruminal bacteria, protozoa and their mixture in vitro. *Anim. Sci. J.***74(1)**: 11-16. doi:10.1046/j.1344-3941.2003.00080.x
- Sylvester JT., Karnati SKR., Yu ZT., Morrison M. and Firkins JL. (2004) Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. *J. Nutr.* **134(12)**: 3378-3384.
- Thioulouse J., Chessel D., Dolédec S. and Olivier JM. (1997) Ade-4: a multivariate analysis and graphical display software. *Stat. Comput.* **7(1)**: 75-83. doi:10.1023/A:1018513530268
- Tomkins NW., Harper GS., Bruce HL. And Hunter RA. (2006) Effect of different post-weaning growth paths on long-term weight gain, carcass characteristics and eating quality of beef cattle. *Aust. J. Expt. Agric.* **46**: 1571-1578.
- Tomkins NW., McGinn SM., Turner DA. and Charmley E. (2011) Comparison of open-circuit respiration chambers with a micrometeorological method for determining methane emissions from beef cattle grazing a tropical pasture. *Anim. Feed Sci. Tech.***166-167**:240-247. doi:10.1016/j.anifeedsci.2011.04.014

9. Appendices

ix.1 Publications arising from the project

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Leucaena (*L. leucocephala*) Based Pastures Have the Potential to Reduce Methane Emissions for Beef Cattle

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Across northern Australia significant areas of Leucaena (>150,000 ha) have been planted to support highly productive livestock systems yielding “grass fed” beef. The area dedicated to Leucaena is expected to exceed 300,000 ha by 2025. Supplementation with Leucaena decreases methane emissions per unit feed intake compared with other tropical legumes (Kennedy and Charmley 2012) and incorporating Leucaena into the diet can result in improved liveweight gain, therefore reducing methane emissions per unit of animal product. The mitigation potential of Leucaena will contribute to abatement methodology development in Australia for ruminant production systems operating in a carbon constrained economy. This project aimed to quantify the greenhouse gas abatement potential of Leucaena pastures.

Thirty *Bos indicus* or cross bred steers were allocated to a grass; Rhodes (*Chloris gayana*), or Leucaena grazing system, double row Leucaena (cv Cunningham) typical of commercial operations across Qld. At 14 -21 d intervals live weight was recorded and animals rotated onto ungrazed pastures. To coincide with methane measurements, rumen fluid was collected by stomach tubing for determination of VFA profile and microbial diversity from 13 to 17 steers per group. An open path laser methodology was used to determine methane emissions at the herd scale (Tomkins *et al* 2011). Metagenomic analysis of the rumen microbial community used next generation sequencing to determine differences in rumen microbiota. High throughput sequencing and barcode “pyrotagging”, phylogenetic based methods targeted the 16S rDNA gene to characterise microbial populations.

Table 1. Mean (\pm s.e) liveweight, average daily gains (ADG), methane, total volatile fatty acid (VFA), and non-Acetic:C2 ratio for steers grazing Leucaena or Rhodes grass pastures throughout 2013

	Month	Liveweight (kg)	ADG (kg/d) ¹	Methane		VFA	
				(g/hd.dsy) ²	(g/kg ADG)	total (mM/L)	non-Acetate : C2 ³
Leucaena	Mar-Apr	409 \pm 7.9	1.09	181.7	166	66.4 \pm 5.87	<i>n.d.</i>
	Jun-July	460 \pm 8.3	0.67	223	332	61.6 \pm 6.60	0.44 \pm 0.008
Rhodes	Mar-Apr	384 \pm 8.0	0.78	250.2	320	64.8 \pm 4.05	<i>n.d.</i>
	Jun-July	423 \pm 8.6	0.5	290.3	580	57.0 \pm 2.84	0.35 \pm 0.005

¹Average daily gain (kg/d) based on 77 and 95 d prior to methane measurements for Mar/Apr, Jun/Jul, respectively;

²estimates generated from bLS modelling using WindTrax software. ³*P* \leq 0.001; *n.d.* not determined. Statistical analysis performed by ANOVA, differences determined by LSD at 5% level (*P* > 0.05). Statistical analyses were run with STATISTICA 6 (StatSoft Inc., Tulsa, Ok.).

Initial results (Table 1) indicate that emissions from cattle with access to Leucaena are up to 27% less than those from cattle grazing Rhodes grass only. Steers with access to Leucaena were approximately 40 kg heavier compared to animals grazing Rhodes grass pastures only. Rumen micro-biomes shared similar bacterial species. Species abundance was affected by the inclusion of Leucaena in the diet. Significant changes were observed for fibrolytic populations; decreases in *Ruminococcus* sp. and concurrent increases in *Fibrobacter* sp. *Prevotella* sp. and *Porphyromonas* sp. were more abundant in Leucaena fed animals and reflect the higher dietary protein content. Potentially it is these species that are responsible for the increase in branched and longer chain fatty acids through enhanced peptide fermentation pathways. An increase in the relative abundance of methanogens belonging to the *Methanoplasmatales* species was associated with Leucaena in the diet. These changes in fermentation and methane forming populations may be the underlying reasons for reduced methane emissions from cattle managed in the Leucaena production systems.

Kennedy P. and Charmley C. (2012). *Anim. Prod. Sci.* 52, 225.

Tomkins N., McGinn S., Turner D. and Charmley, E. (2011). *Anim. Food Sci. & Tech.* 166-167: 240.

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