Final report

Feeding Leucaena to manage the rumen for maximum beef profit

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Abstract

Leucaena leucocephala, a perennial browse legume, represents one of the few nutritional options to significantly improve beef productivity in northern Australia. This project used expert knowledge and existing spatial data sets to map potential distribution in Queensland, the Northern Territory, and northern Western Australia, estimating that up to 27.3M hectares of land in Northern Australia could viably support Leucaena-grass pasture grazing systems. This included 4.6M hectares in humid coastal areas of Queensland that are suitable for the psyllid-resistant Redlands cultivar, with economic analysis of further adoption suggesting a total benefit of $61-123M over the next 40 years across northern Australia.

To maximise the benefits for cattle grazing Leucaena, the cattle need rumen bacteria capable of degrading the toxins mimosine, 3,4 DHP and 2,3 DHP. This project investigated the effect of the Redlands cultivar on the current DAF inoculum resulting in the development of a new mixed bacterial inoculum (TriMix), adapted for better utilisation of the three different Leucaena cultivars, Redlands, Wondergraze and Cunningham. An on-property survey of the presence of de-toxifying bacteria in the rumen found that cattle which had never been exposed to Leucaena did not possess rumen bacteria able to degrade the toxins 3,4 DHP and 2,3 DHP so would benefit from receiving the Department of Agriculture and Fisheries Leucaena inoculum if being moved to a Leucaena-grass pasture grazing system.

Executive summary

Background

Leucaena leucocephala, a perennial browse legume, represents one of the few nutritional options to significantly improve beef productivity in northern Australia. Despite high establishment costs and the risk of establishment failure, the popularity of Leucaena by graziers is driven by superior levels of animal performance and the fact that once established, the crop can persist for many years. Bowen et al., (2016) compared six annual and perennial forages grown under commercial conditions in central Queensland. Their study revealed that a Leucaena-grass system was the most profitable with a gross margin of $181/ha/year compared with the next best system of butterfly pea-grass ($140/ha/year) or annual crops ($18 to $102/ha/year). The superiority of Leucaena was attributed to lower forage costs and higher animal performance. Under more controlled experimental conditions liveweight gains from Leucaena-grass systems are typically around 1 kg/d (Quirk et al., 1988; Harrison et al., 2015) or approximately double that obtained from grass pastures in higher rainfall areas of Queensland.

Despite evidence for Leucaena's long term value in beef production, we have been unable to answer fundamental questions about its use and distribution. A single study used mail surveys to estimate hectares under Leucaena in a small part of eastern Queensland (Lesleighter and Shelton, 1986). Another produced an estimate of the total potential cultivation areas across the state but pre-dated the psyllid resistant Leucaena cultivar "Redlands" or future sterile cultivar that could allow Leucaena to be grown in more humid, psyllid susceptible regions of Queensland and took no account of legislative and code of practice guidelines (Beutel et al., 2018). However, there are quality public spatial datasets that could be used along with expert advice to quantitatively answer questions about where Leucaena is currently established, how much there is, where more could be planted, and how much difference that would make to the beef industry. This is critical information for industry and support agencies attempting to boost the beef industry, through informed use of Leucaena.

To be able to utilise Leucaena efficiently it is necessary to inoculate cattle with bacteria capable of degrading the toxic breakdown products (3,4 DHP, 2,3 DHP) of the toxic amino acid, mimosine, that is prevalent within Leucaena cultivars. Whilst the cultivar Redlands addresses the serious psyllid pest issue, the characteristics that prevent psyllid predation of the plant may also affect the efficacy of the current Department of Agriculture and Fisheries (DAF) Leucaena inoculum and *Synergistes jonesii*, the rumen bacteria that degrades the Leucaena toxins.

The industry needs science-based evidence of the current and potential land areas suitable for planting Leucaena across northern Australia, economic benefits of future adoption of Leucaena-grass grazing systems to use in planning for future extension of beef grazing enterprises in these areas. Science-based evidence of the need to inoculate animals to provide toxin-degrading rumen bacteria and the efficacy of the DAF Leucaena inoculum for animals grazing new cultivars is needed to enable producers to make informed decisions for their animal management practices. This project addresses these issues.

Objectives

The aims of this project were met through the following two objectives:

1) Determine the efficacy of the current and modified Leucaena inoculum for cattle grazing the new psyllid resistant and future sterile cultivars; and

2) Establish the range and extent of land that are suitable for the growth of Leucaena cultivars.

Both objectives were met through:

- the development of a new, TriMix Leucaena inoculum suitable for cattle grazing either the older cultivar or the new psyllid resistant Redlands cultivar, and
- determination of the range and extent of land across northern Australia suitable for growing Leucaena and analysis of potential economic benefits to the Australian beef industry of further adoption.

Methodology

1. A series of 30-day *in vitro* fermentations were undertaken, to determine the effects of the new psyllid-resistant Leucaena cultivar Redlands and psyllid-tolerant cultivar Wondergraze on the bacterial populations in the current Leucaena inoculum. A TriMix Leucaena inoculum was developed in a 30-day *in vitro* fermentation using three starter cultures (Redlands, Wondergraze and Cunningham) and fed leaf from three different cultivars. Daily population numbers of *Synergistes jonesii* were determined using quantitative PCR assays and the ability to degrade toxins, measured in Leucaena toxin degradation assays, set up on days 10, 15, 20, 25 and 30 of each fermentation.

2. Animal ethics approval was obtained to sample cattle for an on-property survey consisting of properties with one of four grazing management treatments: Treatment 1 – a herd which had never been inoculated and were grazing Leucaena; Treatment 2 – a herd received the rumen fluid Leucaena inoculum (pre-1993), with between animal transfer managed and were grazing Leucaena; Treatment 3 - the herd received the DAF Leucaena inoculum, and were grazing Leucaena; Treatment 4 – a herd which had never been inoculated and never grazed Leucaena. Cattle on identified properties were rumen sampled and toxin degradation assays used to determine if cattle possessed toxin-degrading rumen bacteria.

3. Expert knowledge and existing spatial data sets were used to map the potential distribution of cultivated Leucaena (*Leucaena leucocephala* ssp. *glabrata*) in Queensland, the Northern Territory, and northern Western Australia. An economic analysis was undertaken to estimate the economic benefit of further adoption across the study area. The work incorporated separate analyses for the new cultivar Redlands, and all other commercially cultivated cultivars.

Results/key findings

The key findings of the project were:

The psyllid resistant Redlands Leucaena cultivar was found to negatively impact the DAF Leucaena inoculum's ability to degrade two of the toxins, 3,4 DHP and 2,3 DHP, within initial *in vitro* fermentations. To address this loss of activity, a TriMix Leucaena inoculum has been developed containing bacterial populations adapted to effectively ferment and detoxify, plant material from all three different Leucaena cultivars (Cunningham, Redlands and Wondergraze).

The on-property survey results have shown that populations of rumen bacteria able to completely degrade the toxic compounds in Leucaena, are not naturally present in Australian cattle. The rumen bacterial populations in cattle that have never been exposed to Leucaena were unable to degrade the toxins 3,4 DHP or 2,3 DHP. Use of the DAF inoculum to introduce the toxin-degrading bacteria to naïve cattle, is recommended to ensure the maximum benefit from utilising Leucaena-pasture

grazing. The survey showed that these bacteria can be maintained by management practices to ensure its spread to new animals being introduced to Leucaena-pasture grazing.

Mapping estimated up to 27.3M hectares of land in Northern Australia could viably support Leucaena-grass pasture grazing systems. This includes 4.6M hectares in humid coastal areas of Queensland that are suitable for the Redlands cultivar, and where other cultivars have previously been non-viable due to predation from psyllids. The economic analysis suggests a total benefit of $61-123M over the next 40 years across northern Australia. More than 90% of this will be generated in Queensland including $13-26M from cv Redlands cultivation.

Benefits to industry

The benefits for the industry will be:

- The production of the DAF TriMix inoculum, a new mixed bacterial rumen inoculum capable of degrading 3,4 DHP and 2,3 DHP efficiently and effectively, when used in animals grazing different Leucaena cultivars, including the psyllid resistant Redlands;
- Scientific evidence provided by an on-farm survey, on which management practices enable cattle to possess the rumen bacteria able to completely degrade the toxic compounds associated with feeding Leucaena;
- The identification and mapping of land areas in Northern Australia which could viably support Leucaena-grass pasture grazing systems available to enable future expansion; and
- Estimated economic benefits of future expansions to the industry modelled over the next 40 years.

Future research and recommendations

The work on the efficacy of the current DAF Leucaena inoculum resulted in an improved mixed bacterial rumen TriMix inoculum providing bacterial populations adapted to three cultivars which quickly established detoxifying populations in fermentations. The recommendation from this project is for production of the DAF Leucaena inoculum to shift to the TriMix from the next production run.

Future research into the bacteria present in the rumen capable of degrading 3,4 DHP and 2,3 DHP will include rumen microbiome sequencing of the72 animals sampled within the on-property survey and metagenome sequencing of rumen fluid from selected animals. Results and recommendations to industry from on-property survey will be communicated to industry through future planned communication activities including an article in the FutureBeef Newsletter and a FutureBeef or Leucaena Network webinar.

This project established the range and extent of land that is suitable for the growth of Leucaena cultivars and indicates broader areas of northern Australia where industry can scale up planning with agronomic and economic advisors, for best results. This planning could focus on optimal placement of Leucaena at property scale, appropriate weed Leucaena control, and more targeted analysis of potential financial outcomes.

This project suggests the broader areas that would be useful starting points for finer scaled planning involving agronomic and economic advisors. Future projects with extension-based activities focussing on the optimal placement of Leucaena at property scale, appropriate weed control and more targeted analysis of potential financial outcomes to demonstrate the benefits of Leucaena for Northern Australian producers should be undertaken.

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1. Background

Leucaena (*Leucaena leucocephala*) is a leguminous woody plant used for a number of purposes globally. Leucaena leaves have potentially high nutritional value for cattle, and this has resulted in its wide use as a supplementary food for ruminants, including cattle. Cultivation of Leucaena (primarily *L. leucocephala* ssp*. glabrata*) to augment pastures for beef cattle has seen a steady increase in eastern Australia since the 1970s (Beutel et al., 2018). Leucaena represents one of the few nutritional options to significantly improve beef productivity in northern Australia. The benefits include the potential for positive long term financial benefits for the grazing business (Bowen and Chudleigh 2021), as well as potential reductions in cattle greenhouse emissions (Harrison et al., 2015) and nitrogen fixation (Shelton and Dalzell 2007). Leucaena offers the chance for producers to achieve growth rates of over 1 kg/d from their existing land base and research indicates that Leucaena may reduce methane emissions from grazing cattle. Under the emerging carbon economy, it is estimated that Leucaena could generate over $1 million per year in carbon revenue across the north. The superior animal performance, the potential suitable land area, development of psyllid-resistant varieties, and the carbon benefits when taken together, demonstrate a massive potential for Leucaena expansion and finishing systems based on this perennial browse legume.

The psyllid-resistant cultivar “Redlands” is expected to allow Leucaena to be grown in more humid, psyllid susceptible regions of Queensland and this will accelerate additional plantings. Available area is not a limiting factor for expanded adoption of Leucaena-grass systems in northern Australia. Expansion will depend instead on multiple factors including local regulations around issues such as vegetation management and weed Leucaena control, competing land uses, and technical support to ensure better placement, establishment, and productivity. This finding applies to all commercial cultivars including cv Redlands.

Whilst the development of Redlands addresses a serious pest issue, questions were posed regarding the ability of *Synergistes jonesii*, the rumen bacteria that degrades the Leucaena toxins (3,4 DHP, 2,3 DHP) and the efficacy of the current DAF inoculum in relation to the Redlands cultivar. Recently, the need for cattle grazing Leucaena to receive the DAF inoculum to prevent Leucaena toxicity and maximise production potential has been raised. It has been suggested that Australian cattle naturally possess the ability to detoxify mimosine, 3,4 DHP and 2,3-DHP (Shelton et al., 2019). The industry therefore needs science-based evidence of the efficacy of the existing DAF inoculant with new varieties and the distribution of cattle possessing rumen bacteria capable of detoxifying Leucaena toxins.

For successful expansion and establishment of Leucaena pasture production the industry needs accurate, scientifically determined estimates of the current acreage under Leucaena pasture production, and identification of the potential distribution areas and economic benefits of further adoption of cultivated Leucaena in northern Australia. This project addressed these issues.

2. Objectives

Objectives

1) Determine the efficacy of the current and modified Leucaena inoculum for cattle grazing the new psyllid resistant and future sterile cultivars.

This objective has been met successfully. This project determined that, within initial *in vitro* fermentations, the new psyllid resistant cultivars negatively impacted the current DAF Leucaena inoculum's ability to degrade two of the toxins, 3,4 DHP and 2,3 DHP. Re-scoping of the project in January 2022 enabled new research investigating potential improvements to the DAF inoculum. Bacterial populations, adapted to the psyllid resistant cultivars which successfully degraded all toxins in previous fermentations were combined in a fermentation fed leaf from Redlands, Wondergraze and Cunningham cultivars, with the resulting inoculum called TriMix. It was demonstrated that the bacterial populations present in the TriMix inoculum, still established well and retained the ability to effectively degrade toxins, in subsequent fermentations fed leaf from a single cultivar (Redlands, Wondergraze and Cunningham). The TriMix Leucaena inoculum will replace the current DAF inoculum.

2) Establish the range and extent of land that are suitable for the growth of Leucaena cultivars.

This objective has been met successfully with expert knowledge and existing spatial data sets used to map current and potential distribution of cultivated Leucaena in Queensland, the Northern Territory, and northern Western Australia. An economic analysis was undertaken to estimate the economic benefit of further adoption across the study area. The work incorporated separate analyses for the new cultivar Redlands, and all other commercially cultivated cultivars.

3. Methodology

3.1 Efficacy of the current and modified Leucaena inoculum

3.1.1 *In vitro* anaerobic fermentations

To assess the effectiveness of the current commercial Leucaena inoculum to detoxify the Leucaena cultivar Redlands and compare it to the commercially available cultivars Cunningham and Wondergraze, a series of *in vitro* anaerobic fermentations were conducted. Anaerobic fermentations were conducted in a Labfors 3 benchtop fermentation system (Infors HT, Switzerland) using a 3 L fermentation volume. The fermenter vessel was maintained at 39 °C, continuously bubbled with a mixture of CO_2:H_2 (95:5 v/v) to ensure anaerobic conditions and maintained at pH 6.7. Total fermentation time per experiment was 30 days and on the final day of the fermentation, 20 bottles of 'starter cultures' (50 mL of fermenter fluid mixed with 50 mL of the cryopreserving medium RF/Gly (Appendix 12.1.1)) were harvested and stored at -80 °C.

Fermentations were commenced using 3 L of a fermenter starter medium (Appendix 12.1.2) to which was added a 'starter culture' of 100 mL of cryopreserved fermentation fluid. The fermentations, starter cultures and cultivar of Leucaena used as feed, are detailed in Table 3.1. For seven of the 17 fermentations, the starter culture was derived from a commercial inoculum production fermentation (for inoculation of beef cattle fed Leucaena under commercial conditions) and stored at -80 °C. The remaining fermentations had starter cultures which had been collected on the final day of the fermentation.

Initially 40 g of chopped leaf of the corresponding Leucaena cultivar was added as substrate at the commencement of the fermentation. From the second day of fermentation onwards, half of the fermenter liquid volume (i.e. 1.5 L) was removed into 3 x 500 mL Wheaton bottles on a daily basis

and replaced with 1.5 L of fermenter salts solution (Appendix 12.1.3) and 30 g of chopped leaf of the appropriate Leucaena cultivar (Table 3.1) was added.

To develop a commercial inoculum adapted for the detoxification of a range of Leucaena cultivars including Redlands, Wondergraze and Cunningham an *in vitro* anaerobic fermentation (TriMix_Ferm12) was conducted using three starter cultures - Cunningham (Ferm_9, 21/08/2019), Redlands-adapted (Ferm_5, 27/02/2019) and Wondergraze-adapted (Ferm_6, 10/04/2019). The fermentation had equal weights of leaf material of the three Leucaena cultivars - Cunningham, Redlands and Wondergraze, added daily. On day 30, 20 bottles of 'starter culture' were harvested and cryopreserved. A further three separate *in vitro* fermentations were undertaken to determine the ability of the TriMix inoculum to establish and detoxify when fed a single cultivar (Table 3.1). A final *in vitro* fermentation was then conducted using the starter culture harvested from the TriMix_Ferm12 with equal weights of leaf material of the three Leucaena cultivars - Cunningham, Redlands and Wondergraze, added daily.

Table 3.1. Starter culture and feed Leucaena cultivar, for each of the 30-day *in vitro* fermentations, numbered 1 to 17.

	Starter culture	Leucaena cultivar
Ferm_1	Commercial - Cunningham (Bottle #16, 10/12/15)	Redlands
Ferm_2	Commercial - Cunningham (Bottle #5, 10/12/15)	Cunningham
Ferm_3	Commercial - Cunningham (Bottle #6, 10/12/15)	Wondergraze
Ferm_4	Commercial - Cunningham (Bottle #7, 10/12/15)	Redlands
Ferm_5	Redlands-based (Redlands Ferm_1, Day 30)	Redlands
Ferm_6	Wondergraze-based (Wondergraze Ferm_2, Day 30)	Wondergraze
Ferm_7	Commercial - Cunningham (Bottle #9, 10/12/15)	Cunningham
Ferm_8	Commercial - Cunningham (Bottle #12, 10/12/15)	Wondergraze
Ferm_9	Commercial - Cunningham (Bottle #45, 10/12/15)	Cunningham
Ferm_10	Wondergraze-adapted (Wondergraze-based Ferm_6, Day 30)	Wondergraze
Ferm_11	Redlands-adapted (Redlands-based Ferm_5 Day 30)	Redlands
Ferm_12	Redlands-adapted (Ferm_5, 27/2/19) Wondergraze-adapted (Ferm_6, 10/04/19) Cunningham (Ferm_9, 21/8/19)	Cunningham Wondergraze Redlands
Ferm_13	TriMix_Ferm12 (Bottle #2, 15/09/21)	Wondergraze
Ferm_14	TriMix_Ferm12 (Bottle #3, 15/09/21)	Cunningham
Ferm_15	TriMix_Ferm12	Redlands

	(Bottle #4, 15/09/21)	
Ferm_16	TriMix_Ferm12 (Bottle #5, 15/09/21)	Cunningham Wondergraze Redlands
Ferm_17	TriMix_Ferm12 (Bottle #6, 15/09/21)	Cunningham

3.1.2 Fermentation fluid samples for analysis

Samples were collected daily from each fermentation consisting of four 1.0 mL aliquots of fermenter fluid placed into 1.5 mL microcentrifuge tubes, centrifuged at 16,100 *x g* for 10 min, the resulting supernatant was removed and the remaining cell pellet stored frozen at -20 °C for future gDNA extraction and for use as template in *S. jonesii* real time PCR assays. Two 5.0 mL aliquots of fermenter fluid were placed into yellow capped tubes and stored frozen at - 80 °C for future gDNA extraction and microbiome analysis. Six aliquots of 10 mL of fermenter fluid were added to Hungate tubes on days 10, 15, 20, 25 and 30, for Leucaena toxin degradation assays and two 1.0 mL aliquots were placed in cryovials and flash frozen in liquid Nitrogen before being stored frozen at -80 °C for metagenomic or metatranscriptome analyses.

Each day a drop of fermentation fluid was placed on a microscope slide and the bacterial diversity present examined at 400 x magnification on a Nikon Eclipse 80i microscope (Nikon Instruments Inc, Tokyo Japan) and photographed as a reference of the progression of microbial community changes.

3.1.3 Leucaena toxin degradation assay

Leucaena toxin degradation assays were set up on days 10, 15, 20, 25 and 30. The assays for each fermentation were set up with six Hungate tubes each day, under anaerobic conditions and 10 mL aliquots of fermentation fluid added into each tube. The tubes were assigned in duplicate to one of three treatment groups – Mimosine (Mim 1, Mim 2) with 500 µL of a 10 mM mimosine (Simga Aldrich/Merck) solution added to each tube , 3,4-dihydroxypyridine (3,4 DHP 1, 3,4 DHP 2) with 500 µL of a 10 mM 3,4 DHP (prepared 'in-house' from mimosine (Acamovic et al. 1982, Hegarty et al. 1964)) solution added to each tube or 2,3 dihydroxypyridine (2,3 DHP 1, 2,3 DHP 2) with 500 µL of a 10 mM 2,3 DHP (Sigma Aldrich/Merck) solution added to each tube. After mixing by inverting the Hungate tubes, samples were taken immediately (Time 0 h) with a 0.8 mL sample removed using a 1.0 mL syringe and placed into a 1.5 mL microcentrifuge tube. The sample was stored frozen at -20 °C until analysis. The Hungate tubes were incubated at 39 °C with further samples removed at 48 h and 168 h and processed as described for Time 0 h sample.

Quantification of mimosine, 3,4 DHP and 2,3 DHP was performed on a High Performance Liquid Chromatography (HPLC) instrument (Waters, Milford MA USA) with ultra-violet/visible detection (280 nm) following the method described by Tangendjaja and Wills (1980). Briefly, a 200 µL of the thawed sample was filtered through a 0.22 µm syringe-filter and a 10 µL volume loaded onto the HPLC and separated on an Aqua C18 column (Particle size 5 µm, Pore size 125A, 250 x 4.6 mm; Phenomenex) using 0.1% phosphoric acid/ 1% methanol as mobile phase at a flow rate of 1 mL/min. Quantification of mimosine, 2,3-DHP and 3,4–DHP concentrations was determined from comparison to standard curves. A detailed methodology of the toxin HPLC analysis is contained within Appendix 12.2.

3.1.4 Genomic DNA and PCR amplification for *S. jonesii* detection and enumeration

Genomic DNA was extracted from the thawed, pelleted, fermentation fluid using the Repeated Bead Beating and Column (RBB+C) method of Yu and Forster (2005). The quantity and quality of the extracted gDNA was determined by 1% agarose gel electrophoresis in Tris Borate EDTA (TBE) buffer

along with a 5.0 µL aliquot of GeneRuler 1Kb DNA ladder (Thermo Fisher Scientific) and the DNA was visualised using GelRed® stain (Biotium, USA). The extracted gDNA was diluted 1:10 and used as template in two *S. jonesii* specific assays, a conventional PCR and a quantitative PCR.

For the conventional PCR, the *S. jonesii* specific primers sng796F (: 5' TGTGGGGTAAGCAGTTACTC 3') and sng1001R (5' CACCTGTTCTACCTCCTTAGC 3') (McSweeney et al., 1993) were used and each 25 µL reaction contained 10X PCR buffer + 20 mM MgCl2 (2.5 µL); 12.5 µM dNTP mix (0.5 µL); sng796F primer 12.5 pmol/µL (0.5 µL); sng1001R 12 pmol/µL (0.5 µL); ultrapure H_2O (15.4 µL); template DNA (2 µL) and FastStart Taq DNA polymerase 5 U/µL (0.1 µL) (Roche International, Germany). Conventional PCRs were carried out in a C1000 Thermal Cycler PCR machine (Bio-Rad Laboratories Pty., USA) with the hot lid set to 105 °C. The amplification conditions were an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 58 °C for 45 sec, 72 °C for 45 sec, and 95°C for 45 sec; followed by a final extension step of 58 °C for 1 min and 72 °C for 10 min and then held at 12°C until manually stopped. The presence of amplicons was determined by 2% agarose gel electrophoresis in a Tris Acetate EDTA (TAE) buffer at 95 V for 45 min beside a 5.0 µL aliquot of a 100 bp DNA Hyperladder (Bioline, UK), and the DNA was visualised using GelRed® stain.

The *S. jonesii* specific quantitative PCR assay used primers and probe developed in the previous MLA project, B.NBP.0720 - Leucaena Rumen Inoculum – composition and activity along the supply chain, with their sequences listed in Table 3.2. Bacterial cell number standards were prepared as described by Ouwerkerk et al., (2002). In brief, pure cultures of *S. jonesii* were grown in liquid culture and cells counted using a Petroff-Hausser Chamber and diluted in rumen fluid to obtain standards ranging from 10^4 to 10^{10} cells/mL. The gDNA was extracted from these standards using the RBB+C method and stored frozen at -80 °C.

Table 3.2. *S. jonesii* specific quantitative PCR primer and probe sequences

	Sequence (5' to 3')
Sng061F primer	CGAACGGGGATCATGTAGAA
Sng176R primer	ACCTCTCGGCTTATGGGGTA
SngP4 probe	5' 6-FAM CTTAGACATGATTTTAGTGGCGGACGGGT 3' BHQ1*

*6-FAM – 6 caboxyfluorescein fluorescent dye; BHQ1 – black hole quencher 1

The quantitative PCR reactions (25 µL) contained 2.5 µL 10× PrecisionFAST Master Mix reaction buffer (Primerdesign, UK), 200 nM of each primer, 100 nM of oligonucleotide probe and 5 µL DNA template (1:10 dilution of extracted gDNA). PCR was performed in a Rotor-Gene model RG-6000 (Corbett Life Science) using an initial denaturation at 95 °C for 60 sec, followed by a two-step amplification profile consisting of 45 cycles of 95 °C for 15 sec and 60 °C for 30 sec.

3.1.5 Genomic DNA extraction for microbiome analysis

Selected 5.0 mL samples of fermentation fluid microbiome analysis were sent to the laboratory of collaborator Dr Chris McSweeney for processing by his group to minimise variations in gDNA extraction protocols. The initial set of selected samples from Fermentations 1 to 8 were taken across in July 2019 and samples from Fermentation 9 were taken across in August 2019. The gDNA extractions were undertaken by the technician processing the rumen samples taken from animals in the CSIRO methane chamber animal trial.

Briefly, the DNA extraction was carried out on rumen and fermenter samples using the cetyltrimethyl ammonium bromide (CTAB) method of Brookman and Nicholson (2005). Minor modifications as follows: (i) samples were centrifuged (13,000 *x g* for 5 min), and the supernatant was removed before DNA extraction; (ii) cells were homogenized with 200mg of silica–zirconium beads (1:1 mixture of 0.1 and 1.0 mm beads ; Biospec, Bartlesville, OK, USA) and 800 ml of CTAB buffer in a Mini-Beadbeater-8 (Biospec) on maximum speed for 2min, twice; (iii) samples were incubated at 70 °C for 20 min and centrifuged at 10,000 *x g* for 10 min. The supernatant was then

mixed with 500 ml of 25:24:1 phenol–chloroform–isoamylalcohol (Fluka BioChemika, Buchs, Switzerland). The yield and purity of the extracted DNA were assessed with a NanoDrop8000 spectrophotometer (ThermoFisher Scientific, Wilmington, DE, USA).

3.1.6 CSIRO animal trial experimental design

The animal trial associated with this project was undertaken within the CSIRO component of this project at the CSIRO Lansdown Research Station and trial results will be reported by CSIRO separately. The experimental design is summarised in Table 3.3 and provides information regarding the animal numbers, animal diet and experimental grouping as well as the experimental timeline. The labelling of microbiome samples, as used in Section 4.1.2 of this report, are also included. This labelling was intended to describe the diet transitions experienced by animals throughout the duration of the trial (e.g. if the diet of animal 67 was changed from 18% Redlands in periods 1 and 2, to 18% Wondergraze in Periods 3 and 4, the samples were labelled 67_R.W.18).

Table 3.3. Summary of animal trial experimental design and labelling of samples for microbiome analysis, according to the diet transitions experienced throughout the trial.

	Animal	Roughage	Leucaena	Baseline (BL)	Pre1	Post1	Period 1 (P1)	Period 2 (P2)	Pre2	Post2	Period 3 (P3)	Period 4 (P4)	Microbiome analysis diet transition grouping
Group 1:	**67**	0%	0%	All animals on 100% roughage	***Pre-inoculation sampling*** Animals inoculated with 500 mL Leucaena cultivar-specific inoclum	***Post-inoculation sampling*** 3hrs after inoculation			***Pre-inoculation sampling*** Animals inoculated with 500 mL Leucaena cultivar-specific inoclum	***Post-inoculation sampling*** 3hrs after inoculation			67_Control
	30	82%	18%				Redlands	Redlands			Wondergraze	Wondergraze	30_R.W.18
	19	64%	36%				Redlands	Redlands			Wondergraze	Wondergraze	19_R.W.36
	57	52%	48%				Wondergraze	Wondergraze			Redlands	Redlands	57_W.R.48
Group 2:	**22**	0%	0%										22_Control
	48	82%	18%				Wondergraze	Wondergraze			Redlands	Redlands	48_W.R.18
	28	64%	36%				Wondergraze	Wondergraze			Redlands	Redlands	28_W.R.36
	32	52%	48%				Redlands	Redlands			Wondergraze	Wondergraze	32_R.W.48
Group 3:	**26**	0%	0%										26_Control
	27	82%	18%				Redlands	Redlands			Wondergraze	Wondergraze	27_R.W.18
	18	64%	36%				Redlands	Redlands			Wondergraze	Wondergraze	18_R.W.36
	1	52%	48%				Wondergraze	Wondergraze			Redlands	Redlands	1_W.R.48
Group 4:	**9**	0%	0%										9_Control
	65	82%	18%				Wondergraze	Wondergraze			Redlands	Redlands	65_W.R.18
	40	64%	36%				Wondergraze	Wondergraze			Redlands	Redlands	40_W.R.36
	29	52%	48%				Redlands	Redlands			Wondergraze	Wondergraze	29_R.W.48
Sample collection time line			**Group 1**	3/04/2019	24/04/2019		1/05/2019	15/05/2019	22/05/2019		5/06/2019	19/06/2019	
			Group 2	5/04/2019	24/04/2019		3/05/2019	17/05/2019	22/05/2019		7/06/2019	21/06/2019	
			Group 3	10/04/2019	1/05/2019		8/05/2019	22/05/2019	5/06/2019		12/06/2019	26/06/2019	
			Group 4	12/04/2019	1/05/2019		10/05/2019	24/05/2019	5/06/2019		14/06/2019	28/06/2019	

3.1.7 Microbiome amplicon preparation and sequencing

To enable the comparison of the fermentations and rumen microbiomes to be compatible for analysis the preparation of the samples for Illumina sequencing was undertaken at the CSIRO laboratory utilising their customised primer sets. The prepared samples were sent to Macrogen Inc. (Seoul, South Korea) in two separate submissions with the first submission containing the methane chamber animal trial samples and the second submission containing the fermentation samples. This resulted in the samples being next generation sequenced on two separate Illumina MiSeq plates.

3.1.8 Microbiome analysis

To enable the comparison of the fermentations and rumen microbiomes to be compatible, raw Illumina sequence data obtained from Macrogen Inc. (Seoul, South Korea) were pre-processed using the same methodology. Briefly, sequence data was split on the basis of the target primers used in the initial amplicon preparation (primer sets to selectively amplify either bacteria and archaea, archaea, fungi or protozoa) using BBtools (https://jgi.doe.gov/data-and-tools/software-tools/bbtools/bb-tools-user-guide/). Respective sequence datasets were then quality filtered, target primers removed and sequences length trimmed (Cutadapt version 2.8; Marcel, 2011) and further quality filtered using Vsearch (Rognes et al., 2016) to dereplicate, denoise and remove chimeras. The original raw sequence data and the quality filtered data was supplied by CSIRO to DAF and sequence data was archived on the DAF servers and Department of Environment and Science (DES) High Performance Computers (Athena and Apollo).

Four sequence datasets were obtained: (1) fermenter: bacteria; (2) fermenter: archaea; (3) animal trial: bacteria; and (4) animal trial: archaea. All datasets were initially analysed using the Quantitative Insights into Microbial Ecology (QIIME 2) software package (Version 2019.10) (Bokulich et al., 2018; Boylen et al., 2019). Sequence datasets were imported into QIIME 2 and the DADA2 software used for modelling and correcting Illumina-sequenced amplicon errors (Callahan et al., 2016). In this way the input sequences were further quality filtered, the forward and reverse reads merged, unique sequences (sequence variants) grouped, and chimeras removed. A Feature table (the equivalent of the QIIME 1 OTU or BIOM table) containing the counts (frequencies) of each unique sequence (Feature) in each sample in the dataset, a representative sequences file (rep set) and a FeatureData file which maps Feature identifiers in the Feature table to the sequences they represent, was then created. The Feature table was further filtered to remove Features representing < 5 sequences and to remove negative sequencing control samples. A multiple sequence alignment using MAFFT v7 (Katoh and Standley, 2013) and a phylogenetic tree was created to relate Features to one another and assign phylogenetic groups to the Feature table. Taxonomy was assigned using a pre-trained Naïve Bayes classifier trained on the SILVA database (update 132, downloaded 20th February 2019 from QIIME 2 Resources) (Yilmaz et al., 2014; https://www.arb-silva.de/).

As merged read numbers were determined to be relatively low, only the forward reads obtained from CSIRO at a later date (4/02/2020), were used for further analysis. The methods described above were then applied to the single-end datasets to generate Feature tables, representative sequence files, alignment and phylogenetic tree files and to assign taxonomy using the SILVA database (132 update). For the fermenter archaeal dataset, taxonomy was assigned using the specialist, archaea-specific RIM database (Version 14_07; Seedorf et al., 2014). For the fermenter archaea single end (forward read only) dataset, four samples were removed from the analysis due to either the low sequences numbers obtained <2000 sequences (sample Leu2_81) or the irregular taxonomic profiles obtained, resulting in these samples being designated as unexplained outliers: Leu2_87 (Wondergraze fermentation 2, day 30); Leu_78 (Wondergraze adapted fermentation 3, day 25); and Leu2_86 (Wondergraze adapted fermentation 3, day 30). For the animal trial, V4 primer single end (forward read only) sequence dataset, four samples with low sequence numbers were

removed including: Leu1_66; Leu1_118; Leu1_120 and Leu1_119. Analysis of the animal trial dataset was therefore conducted using sequences from the remaining 139 samples.

The taxonomy of specific samples was depicted using taxonomic bar plots generated using QIIME 2, with samples ordered on the x-axis on the basis of specific metadata categories of interest (e.g. Fermentation cultivar and fermentation day). Alpha diversity analysis (microbial diversity within a sample) was determined on the basis of three measures: (1) counts of observed species (Observed Species); (2) Faith phylogenetic diversity (Faith-PD); and (3) Shannon entropy of counts (Shannon).

The three alpha diversity measures were analysed in GenStat v19 (VSN International, 2018) using a repeated measures residual maximum likelihood (REML) method. Day, Fermentation Cultivar and their interaction were fitted as fixed effects with Day within Fermentation Run fitted as a random effect, using a power model covariance structure, to account for the repeated measure over time. Predicted means and standard errors were calculated as well as pairwise comparisons, where significant, using Fishers Protected Least Significant Difference (LSD) at a 5% significance level.

For determination of the differences in the microbial communities occurring between samples (Beta diversity), the respective metadata files, as well as the table, representative sequence (rep set), and unrooted phylogenetic tree (.tre) files generated using QIIME 2, were imported into the R packages, Phyloseq (version 1.30.0; McMurdie and Holmes, 2013; https://joey711.github.io/phyloseq/index.html) and MixOmics (version 6.10.6; Rohart et al., 2017; http://mixomics.org/methods/pls-da/). Statistical exploration and microbial community analysis used a multivariate projection-based approach with repeated measures. For the identification of indicator species and determination of microbial signatures, a sparse Partial Least Squares Discriminant Analysis (SPLSDA) was undertaken. This method was conducted using the MixOmics R package.

Briefly, an unsupervised analysis with Principal Component Analysis (PCA) (Jolliffe, 2005) was conducted using the Feature table data generated using QIIME 2, transformed using the centred log ratio (CLR). To determine the most discriminative Features or OTUs (Features being referred to as OTUs within the MixOmics package), that best characterised factors of interest (e.g. days of each fermentation or each sampling period within the animal trial), a supervised analysis and selection of discriminative OTUs was undertaken with a multivariate analysis SPLSDA on three components (Shen and Huang, 2008; Le Cao et al., 2011). Contribution plots showing the most discriminative OTUs were generated based on the coefficient derived from the component analysis. This indicated the importance of the respective OTUs in determining the microbial signature, with the sign indicating the positive of negative correlations between the OTUs, relative to the proportions of the others. A clustered image heatmap was generated to depict the microbial signatures (OTUs selected from each SPLSDA component) for respective treatment groups (for example, fermentation days).

Core microbial communities were determined following taxonomic classification of Features identified using QIIME 2. Features which were present in 100% of samples according to the metadata category of interest (e.g. day of fermentation), were designated as “core” microbial communities. For comparison of the numbers of core and overall microbial communities present in fermentations maintained on different Leucaena cultivars, the on-line tool Venny was used (https://bioinfogp.cnb.csic.es/tools/venny/; Oliveros, J.C., 2007-2015). This method was used to generate Venn diagrams and lists of microbial populations which were designated as either shared or unique, according to the metadata category of interest (e.g. Leucaena cultivar supplied to the fermenter).

3.1.9 Analysis of Synergistetes populations (sequence alignment and phylogenetic analysis)

For each of the fermenter and animal trial bacterial representative sequence files generated above, a subset of sequences taxonomically assigned to the phylum Synergistetes was created. These sequences were then aligned using Clustal W (Larkin et al., 2007) within Geneious (version 11.1.2, https://www.geneious.com) to determine the presence of single nucleotide polymorphisms (SNPs) and indicate whether representative sequences detected in either the fermenter or animal trial sequence datasets were highly related.

A total of 29 reference 16S rRNA gene nucleotide sequences, representing the major genera found within the phylum Synergistetes were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/; downloaded February, 2020). An unpublished 16S rRNA gene sequence representing the *Pyramidobacter* sp. strain YE332, isolated from the DAF Leucaena fermenter in the MLA project B.NBP.072 (Davis, 2015) was also used as a reference sequence. All reference sequences were aligned with the 15 Synergistetes representative (rep set) sequences obtained from both the fermentations and animal trial datasets, using Clustal W. This alignment was used to trim the reference sequences to the same length and retain only the conserved V4 region of the 16S rRNA gene. The trimmed reference sequences and rep set sequences were then aligned again using Clustal W and this alignment used for all further phylogenetic analysis.

The method used for phylogenetic analysis was selected following a model test (Nei and Kumar, 2000) of 24 different nucleotide substitution models using Mega (version 7; Kumar et al., 2016), with the model with the lowest Bayesian Information Criterion (BIC) score considered to provide the best substitution pattern. In this way, the Kimura 2-parameter model (K2-P) with a discrete Gamma distribution (+G) with 5 rate categories and adopting the assumption that a certain fraction of sites are evolutionarily invariable (+I), was selected for further phylogenetic analysis. A total of 236 nucleotide positions were considered in the final dataset and a Maximum Likelihood phylogenetic tree was generated using the K2-P + G + I model with 1000 replicate bootstraps. The percentage of trees in which the associated taxa clustered together was shown next to the branches and the tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

3.2 On property survey for the prevalence of rumen detoxification of Leucaena toxins

3.2.1 Animal Ethics Approval

An animal ethics application was prepared to undertake survey work to determine the prevalence of rumen organisms capable of degrading the toxins associated with feeding Leucaena, using Leucaena toxin degradation assays. In summary, for the survey, a randomised experimental design was developed with input from DAF Biometrician Dr David Mayer, with four treatments consisting of different production scenarios and the experimental unit being the property. The treatments include:

1. Properties where cattle have never been inoculated with the DAF inoculum for Leucaena detoxification but are grazed on Leucaena.
2. Properties where cattle either received rumen fluid from the original CSIRO cattle or the fistulated cattle held at Brian Pastures Research Station (pre-1993) and have not been inoculated since with the DAF inoculum.
3. Properties where cattle that are grazing Leucaena have been inoculated with the DAF inoculum.
4. Properties that do not have Leucaena and their cattle have never been exposed to Leucaena.

A minimum of three and maximum of five properties/research stations were visited for each production scenario, with samples collected from up to five animals/property. The primary variable was concentrations of the Leucaena-associated toxins mimosine, 2,3-DHP and 3,4-DHP. These degradation assays involved incubating freshly collected rumen fluid with purified toxins to determine the ability of the live rumen bacteria to degrade the three toxins, mimosine, 3,4 DHP and 2,3 DHP rather than just detecting the presence of toxin in the urine or in blood.

3.2.2 On property rumen sampling

Details of the animals (breed, age, sex), the Leucaena cultivar grown (if applicable) and management practice were obtained from the owners. The cattle to be sampled were selected by property owners and mustered to a cattle yard with a crush. The animal to be sampled was restrained in the crush for rumen sampling via a stomach tube with full details of the procedure contained in Appendix 12.3. Samples of rumen fluid were collected for analysis of rumen microbial populations and toxin degradation assays. If possible, collection of faecal material for the proportion of Leucaena being consumed (via faecal NIRS analysis) was obtained by observing the cattle in the yard, race and crush for defecation with collected samples assigned to the animal number and stored frozen at 20 C until analysis.

The collected rumen fluid was filtered through nylon stocking and the following rumen fluid (RF) samples taken and immediately processed on site:

- 6 x 10 mL RF for toxin degradation assays (detailed in section 3.2.3);
- 4 x 1.0 mL RF, centrifuged at 14,000 *× g* in a Mini Spin plus centrifuge (Eppendorf, Hamburg Germany) for 10 min, the supernatant discarded, and the pellet frozen at-18 °C in a 12 V 50 L Kings freezer (Adventure Kings, NSW) run off a 100 Vh lithium battery system for transport back to the laboratory and then stored frozen at -20 °C until analysis. The samples will have DNA extracted for use in *S. jonesii* specific conventional and quantitative PCR assays as detailed in Section 3.1.4; and
- 2 x 1.0 mL RF, centrifuged at 14,000 *× g* in a Mini Spin plus centrifuge for 10 min, the supernatant discarded, and pellet resuspended with 250 µL of RNA protect (Qiagen RNA Protect Bacteria Reagent Cat. # 76506) and frozen at -18 °C in a 12 V 50 L Kings freezer run off a 100 Vh lithium battery system for transport back to the laboratory and then stored frozen at -20 °C until analysis. These samples will be used for reverse transcriptase quantitative PCR assays.

3.2.3 Toxin degradation assay

For the toxin degradation assays to be used for the on-property survey a set of 20 mM toxin standards, consisting of Mimosine, 3,4 DHP and 2,3 DHP, were prepared in the laboratory, aliquoted into 10 mL serum bottles, sealed and stored at 4 °C. Sufficient volumes of each toxin were prepared to use across the anticipated 100 to 120 animals to be sampled.

For each animal sampled, six Hungate tubes (20 mL total volume size) were pre-gassed with a custom anaerobic gas mix (CO_2 97% and H_2 3%) and sealed just prior to the sampling event.

On the day of sampling, as each animal was brought into crush, three sets of duplicate Hungate tubes were labelled with the animal number, date, and the toxin to be assayed before a 0.5 mL aliquot of the appropriate toxin standard was added. The 0.5 mL aliquot was removed from the toxin standard serum bottle using a 1 mL syringe with a 21G needle and added to a pre-gassed Hungate tube through butyl rubber stopper. For each Hungate tube, the lid and butyl rubber stopper were removed and a 10 mL volume of freshly collected rumen fluid (RF) was pipetted in and then immediately resealed. The Hungate tubes were inverted gently several times to mix the toxin standard throughout the RF and a fresh 1 mL syringe and needle was used to remove 0.8 mL of the spiked RF into a 1.5 mL microcentrifuge tube prelabelled with the site, animal number, Time (T0h)

and toxin. This initial, duplicate sample represents the baseline time zero (T0h) of the toxin degradation assay for that animal. The T0h samples were placed in a sealed plastic bag frozen at -18 °C in a 12 V 50 L Kings freezer run off a 100 Vh lithium battery system for transport back to the lab and then stored frozen at -20 °C until analysis. The set of six Hungate tubes for each animal were then placed in a portable incubator (Labec portable incubator #DH2500ABB; power sources AC240V / DC 12V / 12V,40Ah Lithium battery) along with an air temperature data logger (Instrument Devices USB Air Temperature data logger LCD screen, model T-11) for incubation at 39 °C during transportation back to the laboratory. Upon return to the laboratory, the Hungate tubes were transferred to the laboratory 39 °C incubator.

After 48 h of incubation the Hungate tubes were removed, 0.8 mL of the spiked RF sample (T48h) was removed as described previously and stored frozen at -20 °C until analysis. The Hungate tubes were returned to the incubator and after 168 h incubation a final 0.8 mL sample (T168h) was taken from each of the Hungate tubes and stored frozen at -20 °C until analysis.

For analysis of toxin levels, the 0.8 mL samples were thawed and centrifuged in an Eppendorf benchtop centrifuge (Eppendorf, model 5415R) at 16,000 × *g* for 5 min at room temperature. The resulting supernatant was removed and filtered through a 0.45 µm Spin-X microcentrifuge filter (Costar Cat # 8170) prior to HPLC analysis as described in Section 3.1.3.

3.3 Potential distribution and economic benefits of cultivated Leucaena in northern Australia

This work examined the potential extent and economic benefit of cultivated Leucaena (*Leucaena leucocephala* ssp. *glabrata*) in northern Australia. We used expert knowledge and existing spatial data sets to map potential distribution in Queensland, the Northern Territory, and northern Western Australia. An economic analysis was conducted to estimate the economic benefit of further adoption across the study area. The work incorporated separate analyses for a new psyllid tolerant cultivar (cv Redlands) and all other commercially cultivated cultivars. All methods are described in detail in the accompanying report, *The potential distribution and economic benefits of cultivated Leucaena in northern Australia* (Appendix 12.6).

4. Results

4.1 Efficacy of the current and modified Leucaena inoculum

4.1.1 *In vitro* inoculum studies

The current DAF inoculum is produced using leaf harvested from the Leucaena cultivar Cunningham grown at Brian Pastures Research Station. To investigate the efficacy of the current DAF inoculum in detoxifying mimosine and its toxic degradation products (3,4 DHP and 2,3 DHP), when fed the new Leucaena cultivar Redlands, compared to the cultivars Wondergraze and Cunningham, a series of 11 *in vitro* fermentations of 30-day duration were undertaken.

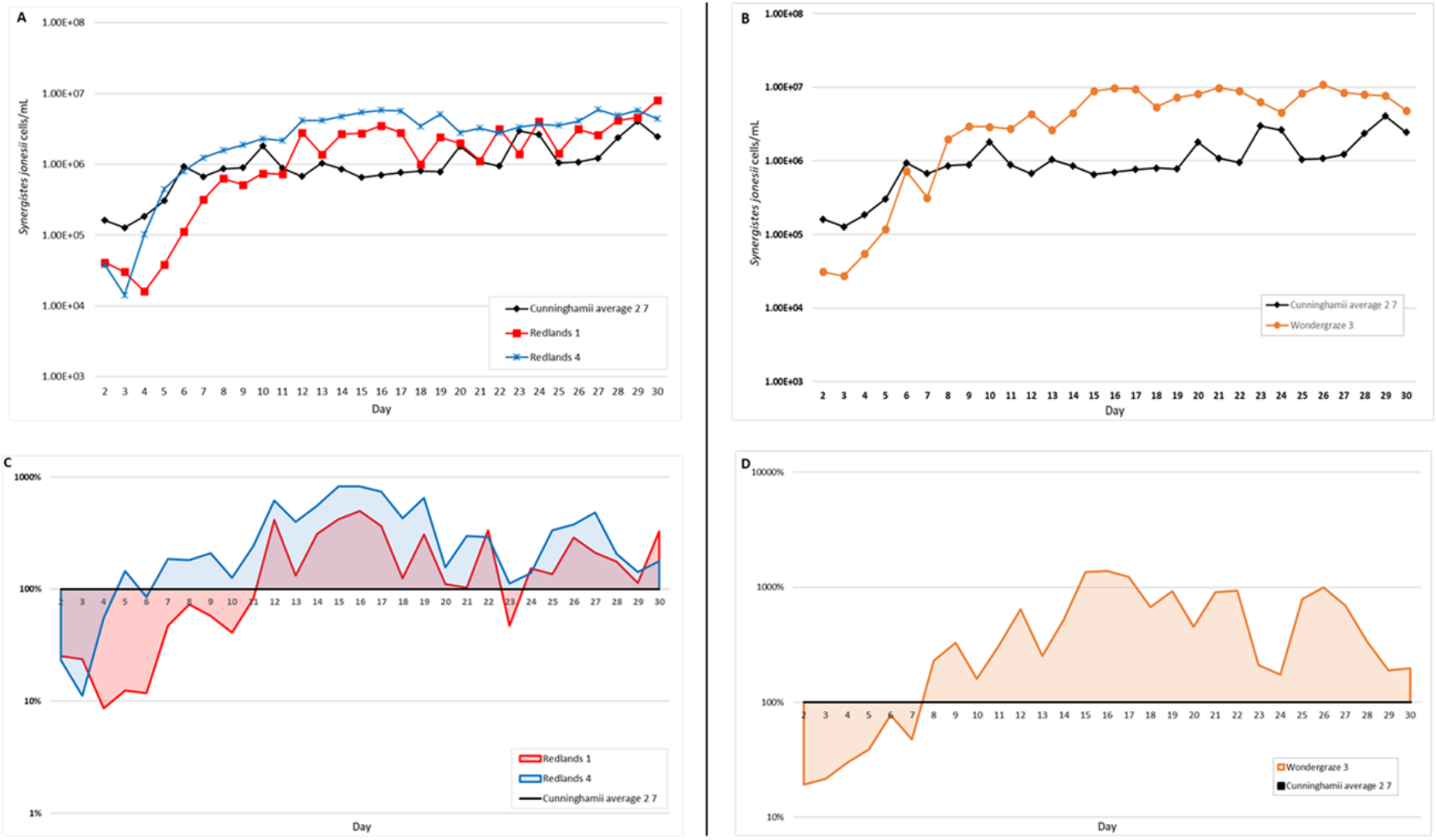
Six of the 11 fermentations (Ferm_1 to Ferm_4, Ferm_7 to Ferm_9) were started using the commercial Leucaena inoculum starter cultures and were then fed Leucaena leaf material from a single Leucaena cultivar (Cunningham, Redlands or Wondergraze). The daily microscopic examination of a drop of fermentation fluid showed similar diversities of bacteria present in all six fermentations. An issue with the temperature probe used in the Infors fermentation system was detected during Ferm_9 (DAF inoculum starter, Cunningham leaf) resulting in the actual temperature in the vessel being outside of the temperature alarm parameters whilst the control panel was displaying the appropriate temperature. Subsequent analyses of data from Ferm_8 (DAF inoculum starter, Wondergraze leaf) and Ferm_9 showed serious impacts on *S. jonesii* populations so the results from these two fermentations were discarded.

4.1.1.1 Commercial DAF inoculum fermentations

In the two fermentations started with the commercial DAF inoculum and fed leaf from the Redlands cultivar and the fermentation fed leaf from the Wondergraze cultivar, the *S. jonesii* populations, measured by quantitative PCR assay, were compared with the average of Cunningham fed Ferm_2 and Ferm_7 daily *S. jonesii* populations (Fig. 4.1 A, B). Using the average number of *S. jonesii* cells present in the Cunningham Ferm_2 and Ferm_7 as the reference point in Fig. 4.1 C, D (100% black line), the numbers present each day in the Redlands and Wondergraze fermentations are visualised as either under (lower cell numbers) or over (higher cell numbers) this line. This clearly shows the negative effect of the Redlands cultivar on *S. jonesii* cell numbers when the fermentations were started with the commercial DAF inoculum starter culture and demonstrates the recovery of *S. jonesii* populations over the 30 days. Initially the *S. jonesii* populations dropped to 10% of what was present when Cunningham leaf was fed with populations taking 5 to 10 days to return to levels similar to the Cunningham fed fermentations (Fig. 4.1 C). The populations adapted and by the final day of the fermentations were present at levels higher than the Cunningham fed fermentations. Starter cultures were harvested on the final day of the fermentations and used to commence 'Redlands-adapted' Ferm_5.

In the Wondergraze fermentation a similar, but less severe, impact on *S. jonesii* cell numbers was seen. Initially the *S. jonesii* populations dropped on day 3 of the fermentation to approximately 30% of what was present on day 3 when Cunningham leaf was fed (Fig. 4.1 D). The *S. jonesii* populations recovered faster starting to increase on day 4 and by day 7 present at levels comparable to the Cunningham fed fermentations. This indicates the bacterial communities in the fermentation adapted to the Wondergraze leaf as a feed substrate and by the final day of the fermentation *S. jonesii* populations were present at levels higher than seen in the Cunningham fed fermentations. Starter cultures were harvested on the final day of the fermentations and used to commence 'Wondergraze-adapted' Ferm_6 and Ferm_10.

Figure 4.1. Effect of feeding different Leucaena cultivars on the populations of *S. jonesii* in 30-day fermentations started with commercial Leucaena inoculum. Daily *S. jonesii* numbers determined by quantitative PCR in fermentations fed either Redlands (A) or Wondergraze (B). The average of *S. jonesii* numbers in the Cunningham Fermentation 2 and Fermentation 7 as 100% (black line), the numbers present each day in the other fermentations are shown as a percentage compared to this, for fermentations fed either Redlands (C) or Wondergraze (D).



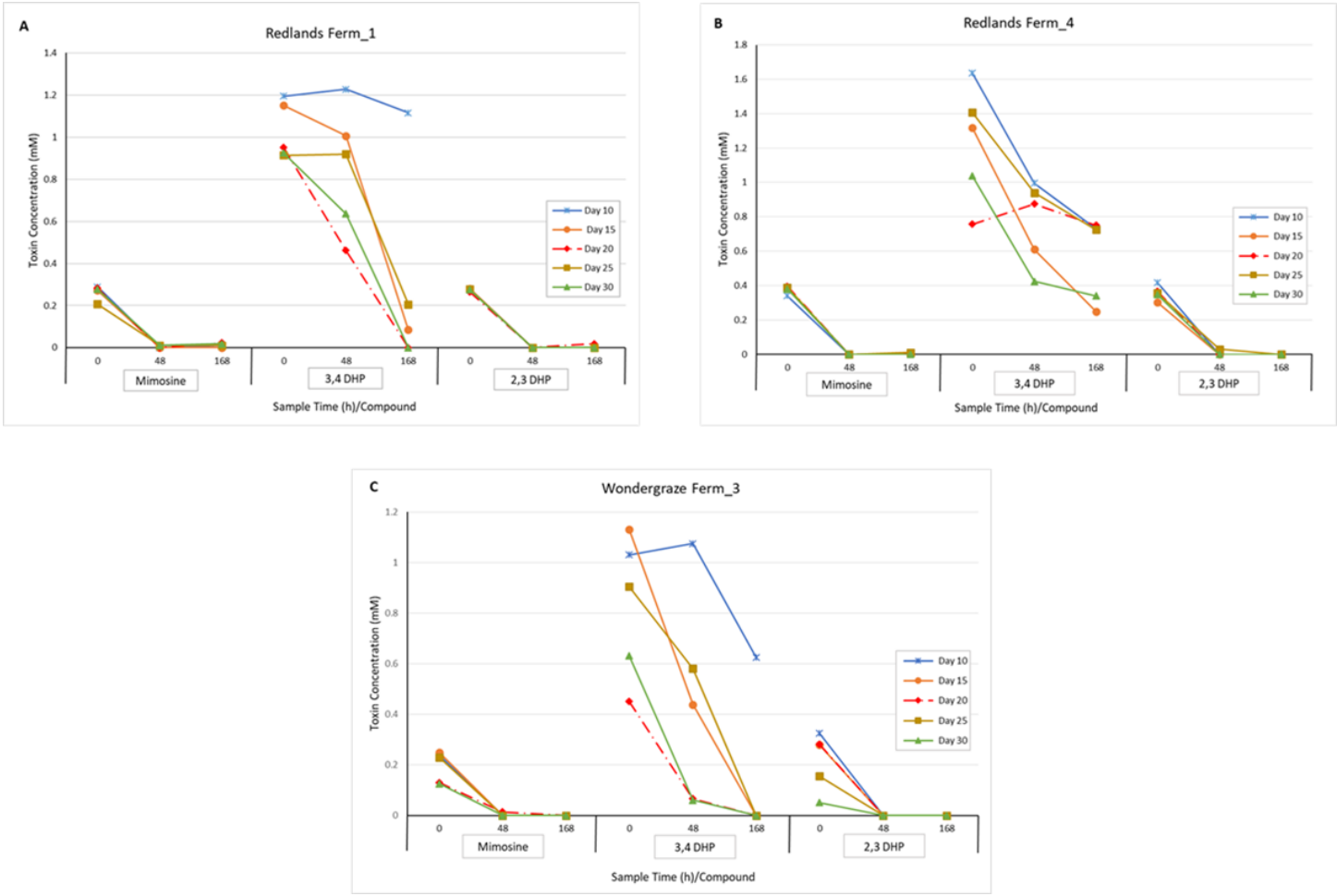
In the three fermentations started with commercial Leucaena inoculum and fed either Redlands or Wondergraze leaf, to track the microbial degradation of mimosine, 3,4 DHP and 2,3 DHP, Leucaena toxin degradation assays were set up every five days from day 10 to day 30. All three fermentations contained bacterial populations that were able to completely degrade mimosine and 2,3 DHP within 48 h from day 10 onwards. However, neither of the Redlands fed fermentations were able to completely degrade 3,4 DHP at day 10 over 168 h (Fig. 4.2 A, B). The Redlands Fermentation 4 did not completely degrade 3,4 DHP by Time 168 h in any of the five degradation assays (Fig. 4.2 B).

The bacterial populations present in the Wondergraze fed fermentation did not completely degrade 3,4 DHP at day 10 over 168 h, however by day 15 they had adapted to digest the Wondergraze leaf material provided and completely degraded 3,4 DHP in all remaining toxin degradation assays (Fig. 4.2 C).

The bacterial populations present in the fermentations were able to adapt to compounds present in the psyllid-resistant Redlands or psyllid tolerant Wondergraze and populations able to degrade the toxin 3,4 DHP recovered over the 30 days of the fermentations. However, this initial drop in the bacterial populations over the first seven to ten days may mean they don't establish well when drenched into animals eating these cultivars.

To determine if the adapted bacterial populations would establish faster using starter cultures harvested on the final day of cultivar-adapted fermentations, a series of four further fermentations were undertaken.

Figure 4.2. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h from toxin degradation assays set up every five days from day 10 to day 30 for A. Redlands Fermentation 1 (Ferm_1) and B. Redlands Fermentation 4 (Ferm_4); and C. for Wondergraze Fermentation 3 (Ferm_3).



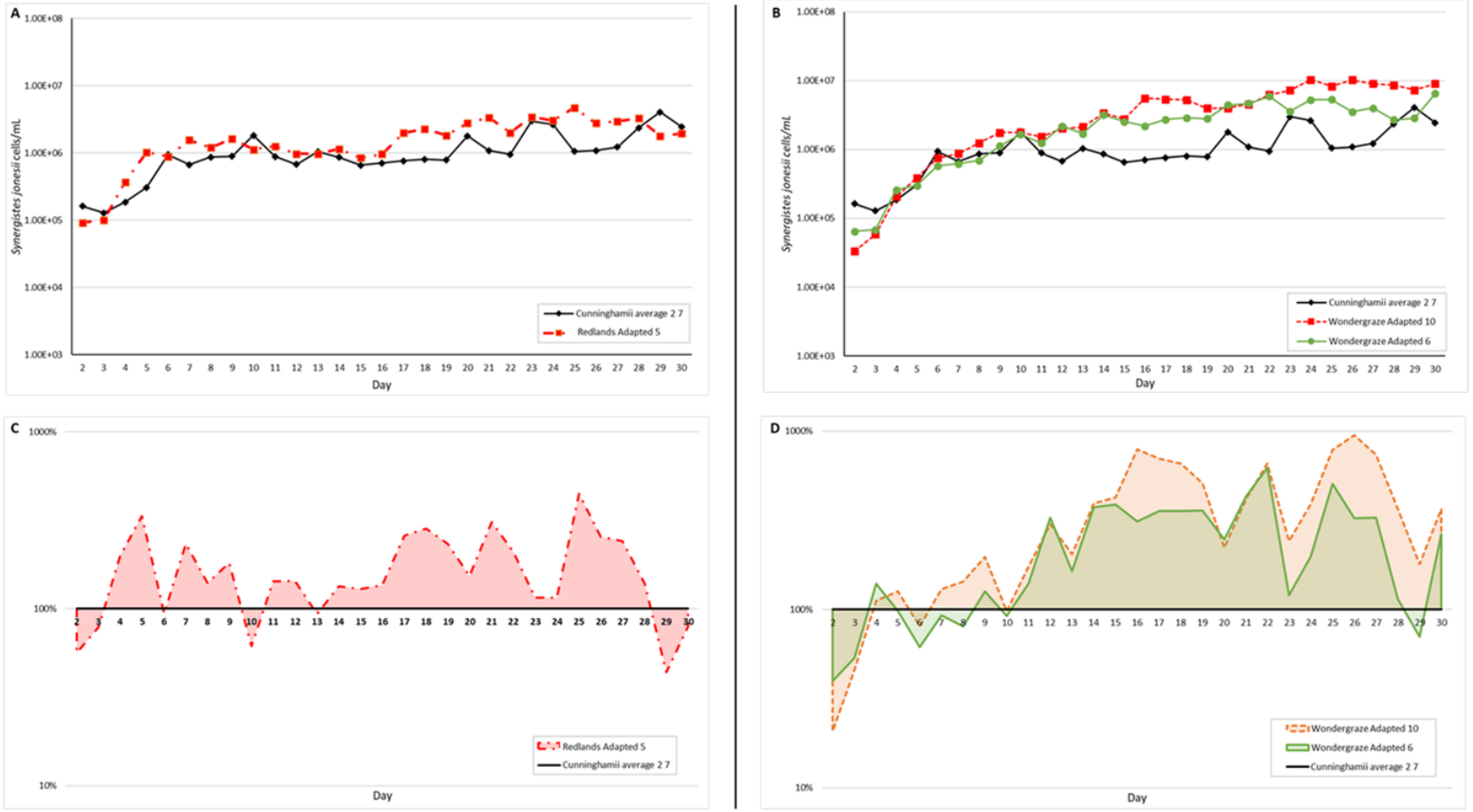
4.1.1.2 Leucaena cultivar-adapted fermentations

To evaluate the toxin degradation activity of bacterial populations that appeared to have adapted to the Redlands or Wondergraze leaf as feed substrate, a fermentation (Ferm_5) run was started using day 30 harvested starter cultures from Fermentation 1 (Ferm_1) and fed Redlands leaf material. In Ferm_5 the *S. jonesii* populations present on day 2 were lower than the average numbers in the Cunningham fermentations but the populations immediately started to increase (Fig 4.3 A, B). Fermentation fluid was harvested from day 20 through to the end of the fermentation and cryopreserved as 'Redlands-Adapted' inoculum.

A second Redlands-adapted fermentation (Ferm_11) was undertaken however oxygen contamination in the anaerobic gas supply was detected during the fermentation. After the completion of the 30-day fermentation, the gas issues were subsequently determined to have adversely affected the fermentation and the data was discarded.

Two further fermentations (Ferm_6, Ferm_10) were undertaken to evaluate the bacterial populations that had adapted to the Wondergraze cultivar and these were started using day 30 harvested starter cultures from Ferm_3 and both fed Wondergraze leaf. The *S. jonesii* populations present in the fermentations on day 2 were lower than the average numbers in the Cunningham fermentations but immediately started to increase (Fig 4.3 C, D). Fermentation fluid was harvested from day 20 through to the end of the fermentation and cryopreserved as 'Wondergraze-Adapted' inoculum.

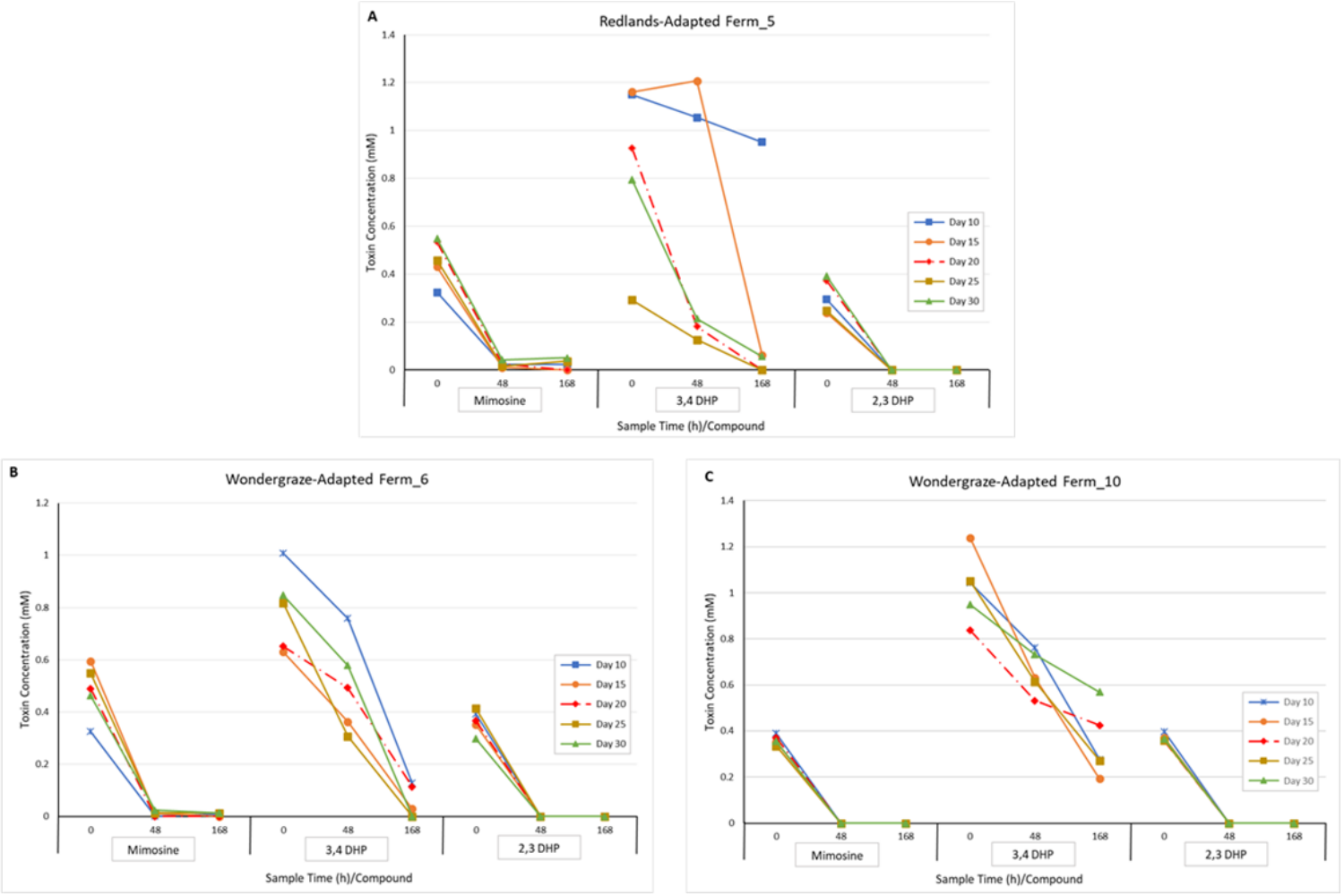
Figure 4.3. Effect of feeding different Leucaena cultivars on the populations of *S. jonesii* in 30-day fermentations started with starter culture harvested from the final day of fermentations fed either Redlands or Wondergraze and designated as adapted fermentations. Daily *S. jonesii* numbers determined by quantitative PCR in adapted fermentations fed either Redlands (A) or Wondergraze (B). The average of *S. jonesii* numbers in the Cunningham fermentations 2 and 7 as 100% (black line), the numbers present each day in the other fermentations are shown as a percentage compared to this, for adapted fermentations fed either Redlands (C) or Wondergraze (D).



To track the ability of the Redlands-adapted Ferm_5 bacterial populations to degrade mimosine, 3,4 DHP and 2,3 DHP, Leucaena toxin degradation assays were set up every five days from day 10 to day 30. The bacterial populations present in the fermentation were able to completely degrade mimosine and 2,3 DHP within 48 h from day 10 onwards (Fig. 4.4 A). In the day 10 degradation assay they were not completely degrading 3,4 DHP over the 168 h of incubation, however in the remaining degradation assay from day 15 onwards they were completely degrading 3,4 DHP (Fig. 4.4 A).

For the Wondergraze-adapted bacterial populations, present in Ferm_6 and Ferm_10 Leucaena toxin degradation assays were set up every five days from day 10 to day 30. The bacterial populations present in the fermentation were able to completely degrade mimosine and 2,3 DHP within 48 h from day 10 onwards(Fig. 4.4 A, B). Neither fermentation was able to completely degrade 3,4 DHP at day 10 over 168 h, however, by day 15 onwards the Ferm_6 bacterial populations were completely degrading 3,4 DHP (Fig. 4.4 A,). The bacterial populations in Ferm_10 did degrade the majority of the 3,4 DHP in day 15 to 25 degradation assays but in the day 30 only 30% of the 3,4 DHP was degraded after 168 h of incubation (Fig. 4.4 B). One possible explanation for the decrease in 3,4 DHP degradation in Ferm_10 may be that the oxygen contamination of the anaerobic gas supply, detected during the Redlands-Adapted Ferm_11 which adversely affected the fermentation resulting the fermentation's data being discarded, may have originally occurred during Ferm_10.

Figure 4.4. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h from toxin degradation assays set up every five days from day 10 to day 30 for A. Redlands-Adapted Fermentation 5 (Ferm_5); B. Wondergraze-Adapted Fermentation 6 (Ferm_6); and C. for Wondergraze-Adapted Fermentation 10 (Ferm_10).



4.1.1.3 Cultivar-adapted inoculum supply for animal trials

A meeting with CSIRO collaborators Dr Ed Charmley and Dr Chris McSweeney was held in January 2019, to discuss the supply of inoculum for the cattle pen feeding trials to quantify the reduction in methane emissions by cattle fed either Redlands or Wondergraze. Following this, the decision was made to supply bottles of inoculum harvested from day 20 through to day 30 of a 'Redlands Adapted' and a 'Wondergraze Adapted' fermentation. A variation was made within the animal trial, whereby the animals were moved onto the alternative Leucaena cultivar at the halfway point of the experiment. Following further discussions, it was decided to re-inoculate the animals with the cultivar-adapted inoculum matching the Leucaena cultivar they would be moving onto at the change over time point. This resulted in double the amount of inoculum originally required; with 36 bottles of frozen inoculum, consisting of 18 bottles of 'Redlands Adapted' and 18 bottles of 'Wondergraze Adapted', sent to Townsville CSIRO in April 2019.

Ongoing lack of rain at the CSIRO Lansdown Research Station meant that the stands of Wondergraze and Redlands leucaena cultivars had insufficient growth to support grazing by animals within the CSIRO grazing trial planned to commence in late 2019. Rainfall in January 2020 resulted in a flush of growth and 32 bottles of frozen inoculum, consisting of 18 bottles of 'Redlands Adapted' and 18 bottles of 'Wondergraze Adapted', were freighted to Townsville CSIRO in February 2020. The grazing trial was impacted by the start of the COVID-19 pandemic resulting in no rumen samples being collected for microbiome work. A replacement CSIRO grazing trial commenced in 2021 and a further 24 bottles of inoculum consisting of 12 bottles of 'Redlands Adapted' and 12 bottles of 'Wondergraze Adapted', were provided to CSIRO in January 2021.

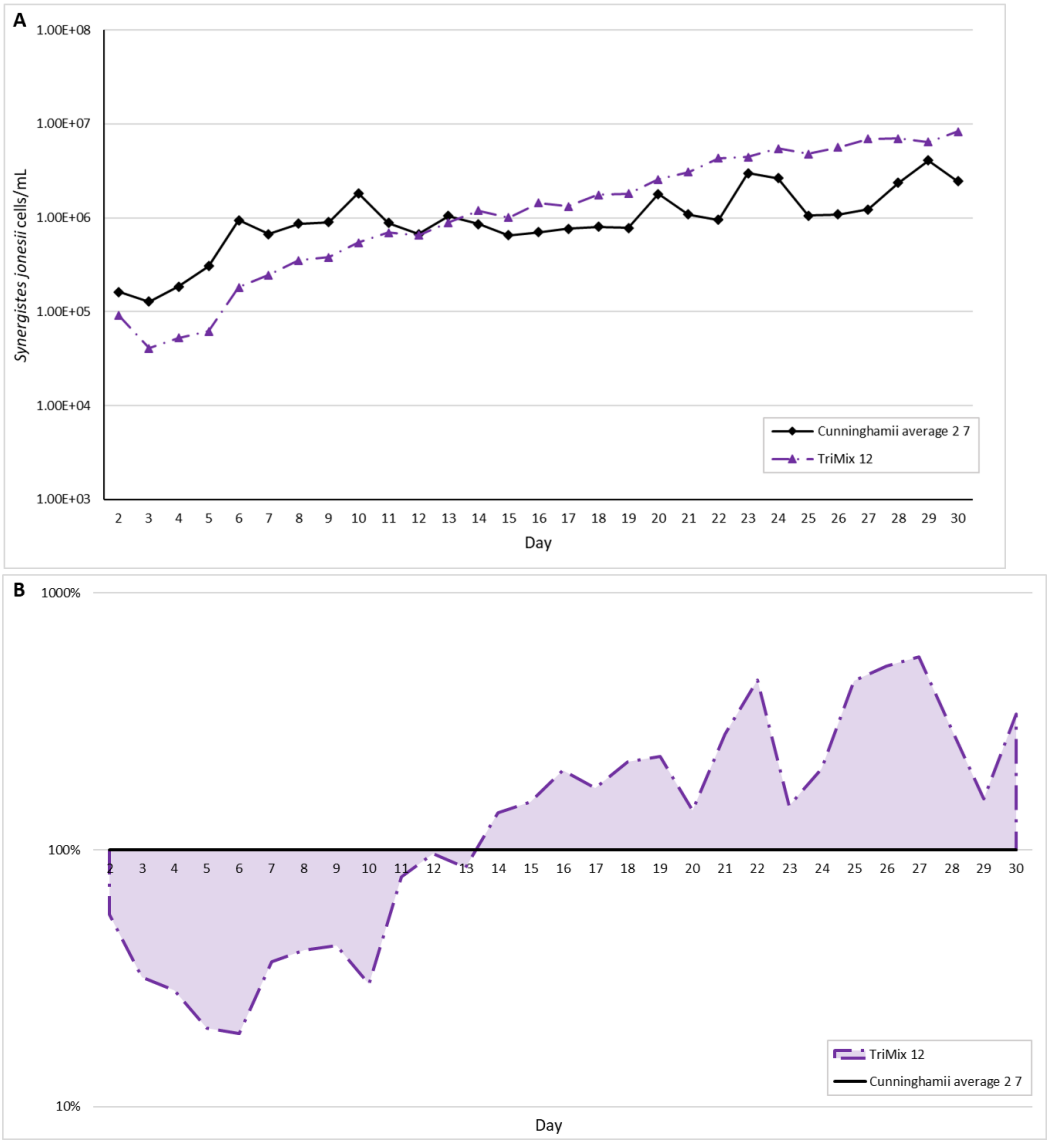
For the DAF run Redlands and Wondergraze comparison animal grazing trial, undertaken at Pinnarendi Station, 12 bottles of frozen inoculum consisting of 6 bottles of 'Redlands Adapted' and 6 bottles of 'Wondergraze Adapted', were provided to DAF Mareeba in March 2020.

4.1.1.4 TriMix inoculum development

To investigate potential improvements in the production of the DAF inoculum a fermentation, (TriMix_12), was undertaken. The 30-day fermentation had three starter cultures (DAF commercial inoculum, Redlands-adapted, Wondergraze-Adapted) added on day 1 and was fed daily with equal amounts of leaf material from Redlands, Wondergraze and Cunningham Leucaena cultivars. Starter cultures were collected on the final day of the fermentation, and these were used to start four subsequent fermentations, three of which were fed leaf material from a single cultivar and a fourth fermentation fed equal amounts of the three cultivars.

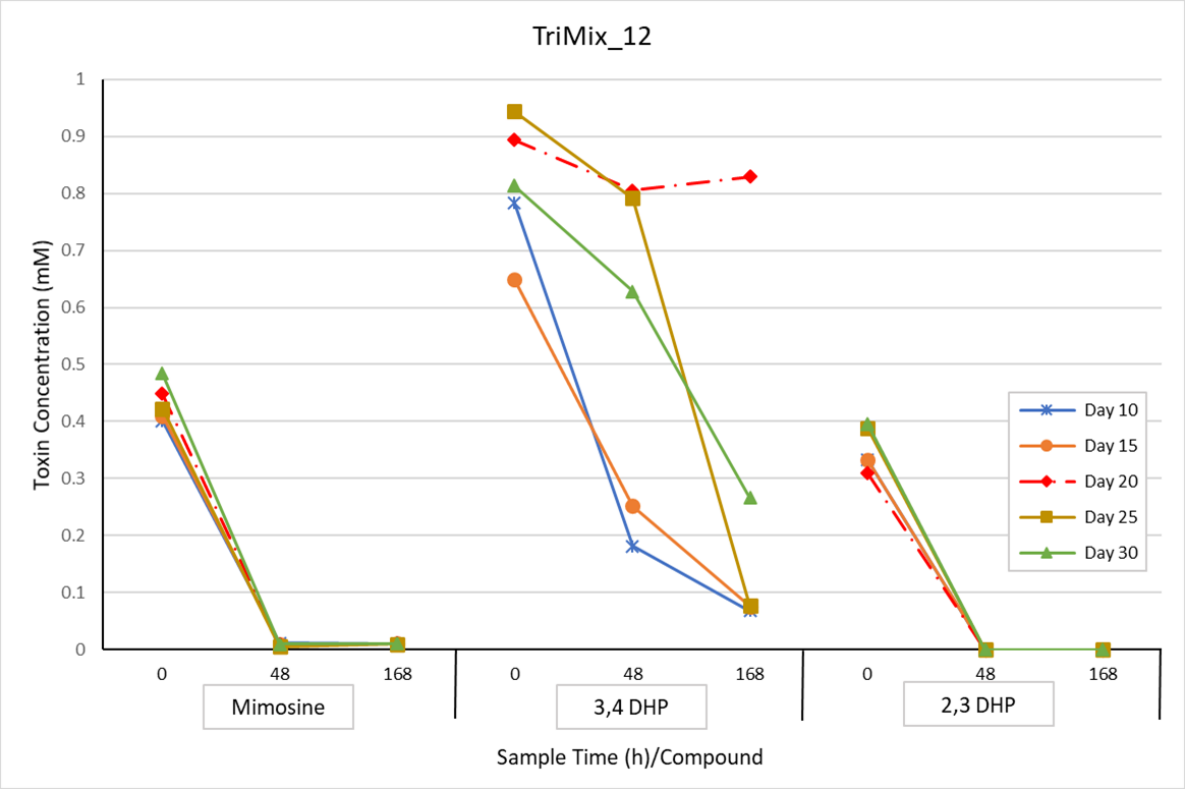
In the initial TriMix_12 fermentation the populations of *S. jonesii* present in daily samples collected across the 30 days, determined using a *S. jonesii* specific quantitative PCR assay, showed an initial dip in population numbers during the first five days of the fermentation, but by day 20 the *S. jonesii* numbers were comparable to the other fermentations (Fig. 4.5 A, B).

Figure 4.5. Effect of using three different starter cultures and feeding equal amounts of Cunningham, Redlands and Wondergraze leaf on the populations of *S. jonesii* in a 30-day fermentation. A. Daily *S. jonesii* numbers determined by quantitative PCR. B. Using the average of *S. jonesii* numbers in the Cunningham Fermentations 2 and 7 (Ferm_2 and Ferm_7) as 100% (black line), the numbers present each day in the Trimix Fermentation 12 (Trimix_12) are shown as a percentage compared to this.



The bacterial populations present in the TriMix_12 fermentation degraded mimosine and 2,3 DHP completely in toxin degradation assays from day 10 onwards (Fig. 4.6). In the day 10 and 15, 3,4 DHP degradation assays, the toxin was completely degraded. However, in the day 20 assay the 3,4 DHP was not degraded and then degraded again in the day 25 and 30 assays (Fig. 4.6). This result was unusual and may have been a technical error as on day 10 the populations of *S. jonesii* were only present at approximately 30% as seen in the Cunningham fermentations.

Figure 4.6. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h from toxin degradation assays set up every five days from day 10 to day 30 for the TriMix Fermentation 12 (TriMix_12).



The subsequent TriMix_Wondergraze_13 fermentation, which was inoculated with a starter culture collected on day 30 of the TriMix_12 fermentation added on day 1, was fed Wondergraze leaf. The populations of *S. jonesii* present in daily samples collected across the 30 days, determined using a *S. jonesii* specific quantitative PCR assay, increased from day 2 onwards (Fig. 4.7 A) and had higher population numbers present throughout the run compared to the average of the Cunningham fed fermentations (Fig. 4.7 B).

The TriMix_Redlands_15 fermentation, was also inoculated with a starter culture collected on day 30 of the TriMix_12 fermentation added on day 1 but was then fed Redlands leaf. The populations of *S. jonesii* present in the fermentation increased from day 2 onwards (Fig. 4.7 C). Comparing the *S. jonesii* populations to the average in Cunningham fed fermentations that had DAF inoculum starter cultures added on day 1, numbers were higher only dropping to lower levels on the last two days of the fermentation (Fig. 4.7 D).

The TriMix_Cunningham_14 and repeat Trimix_Cunningham_17 fermentations, were inoculated with a starter culture collected on day 30 of the TriMix_12 fermentation, added on day 1 and then fed Cunningham leaf. The populations of *S. jonesii* present in the fermentations increased from day 2 onwards (Fig. 4.8 A) and had higher population numbers compared to the average of the Cunningham fed fermentations that had DAF inoculum starter cultures added on day 1 (Fig. 4.8 C).

The final fermentation of the series, TriMix_TriMIx_16 was a follow-on fermentation which was inoculated with a starter culture collected on day 30 of the TriMix_12 fermentation, added on day 1 and was fed equal amounts of leaf from all three cultivars. The populations of *S. jonesii* present in the fermentation increased from day 2 onwards (Fig. 4.8 B) and had higher population numbers compared to the average of the Cunningham fed fermentations that had DAF inoculum starter cultures added on day 1 (Fig. 4.8 D).

Figure 4.7. Effect of feeding different Leucaena cultivars on the populations of *S. jonesii* in 30-day fermentations started with starter culture harvested from the final day of the Trimix fermentation. Daily *S. jonesii* numbers determined by quantitative PCR in TriMix Fermentations 13 and 15, fed either Wondergraze (A) or Redlands (B), respectively. The average of *S. jonesii* numbers in the Cunningham Fermentations 2 and 7 (Ferm_2 and Ferm_7) as 100% (black line), the numbers present each day in the other fermentations are shown as a percentage compared to this, for adapted fermentations fed either Wondergraze (C) or Redlands (D).

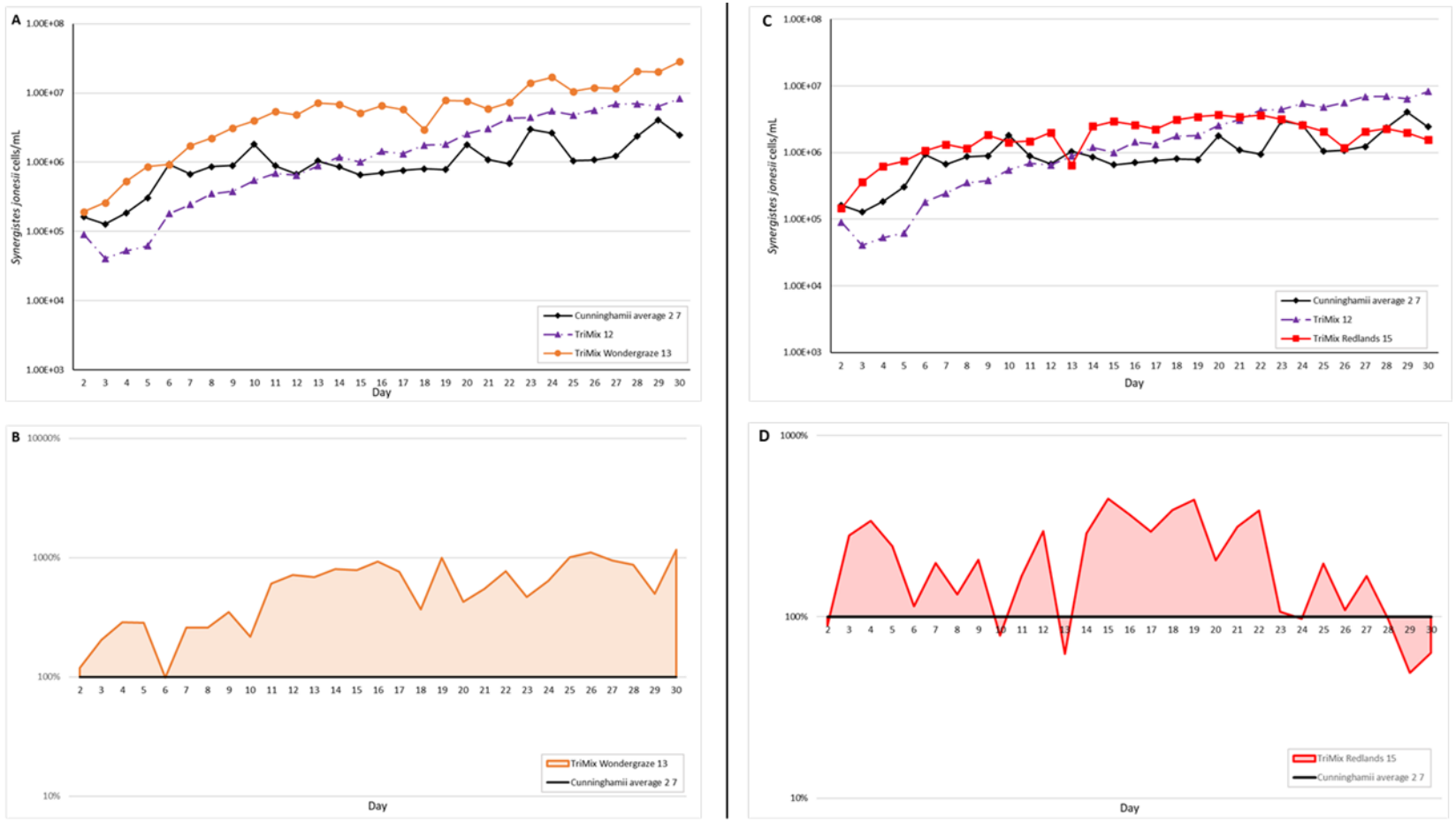
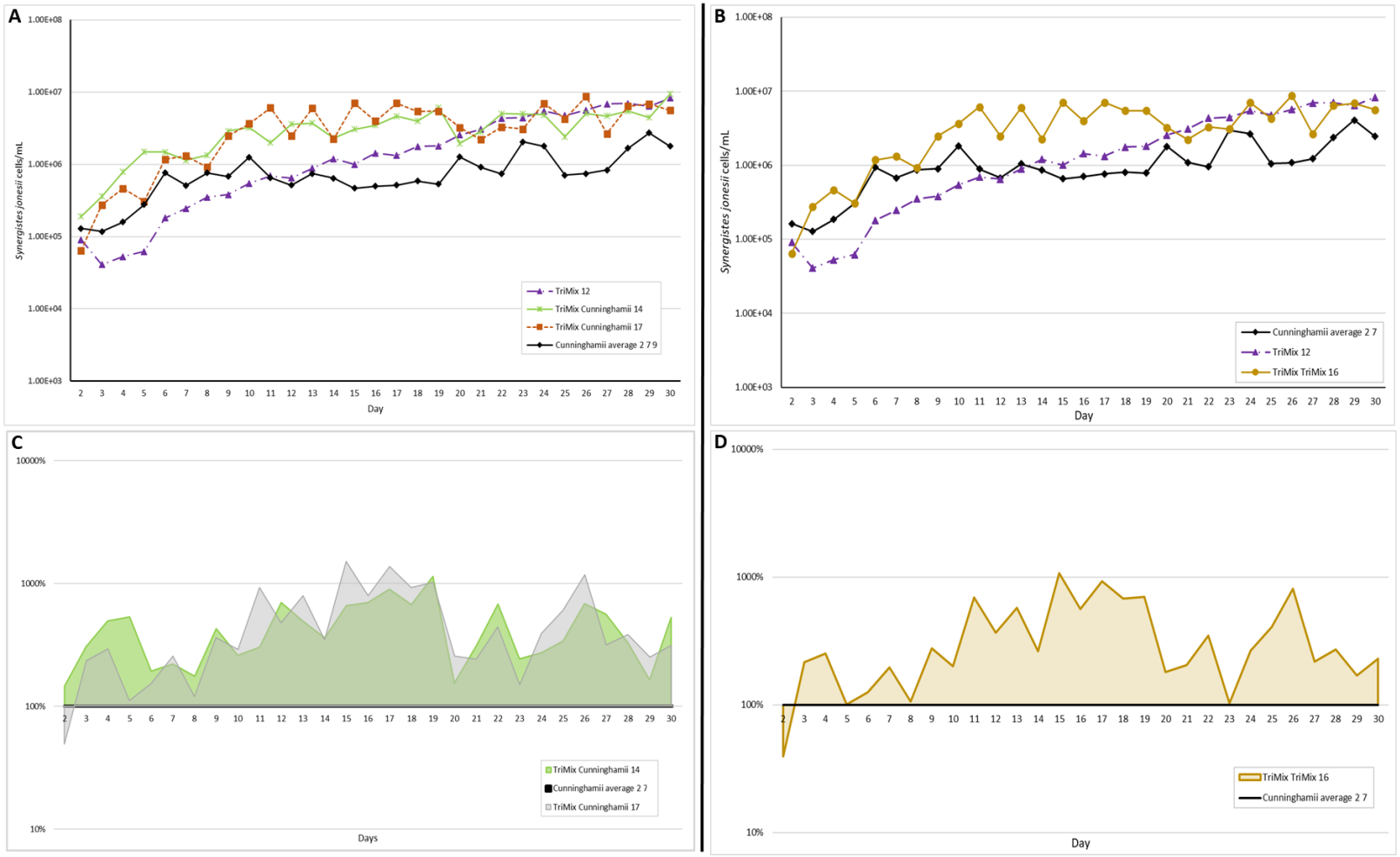


Figure 4.8. Effect of feeding different Leucaena cultivars on the populations of *S. jonesii* in 30-day fermentations started with starter culture harvested from the final day of the Trimix fermentation. Daily *S. jonesii* numbers determined by quantitative PCR in TriMix fermentations fed either Cunningham (A) or TriMix leaf combination (B). The average of *S. jonesii* numbers in the Cunningham Fermentations 2 and 7 (Ferm_2 and Ferm_7) as 100% (black line), the numbers present each day in the other fermentations are shown as a percentage compared to this, for adapted fermentations fed either Cunningham (C) or TriMix leaf combination (D).



The bacterial populations present in both the TriMix_Wondergraze_13 and TriMix_Redlands_15 fermentations were capable of completely degrading mimosine, 3,4 DHP and 2,3 DHP in toxin degradation assays from day 10 onwards (Fig. 4.9 A, B).

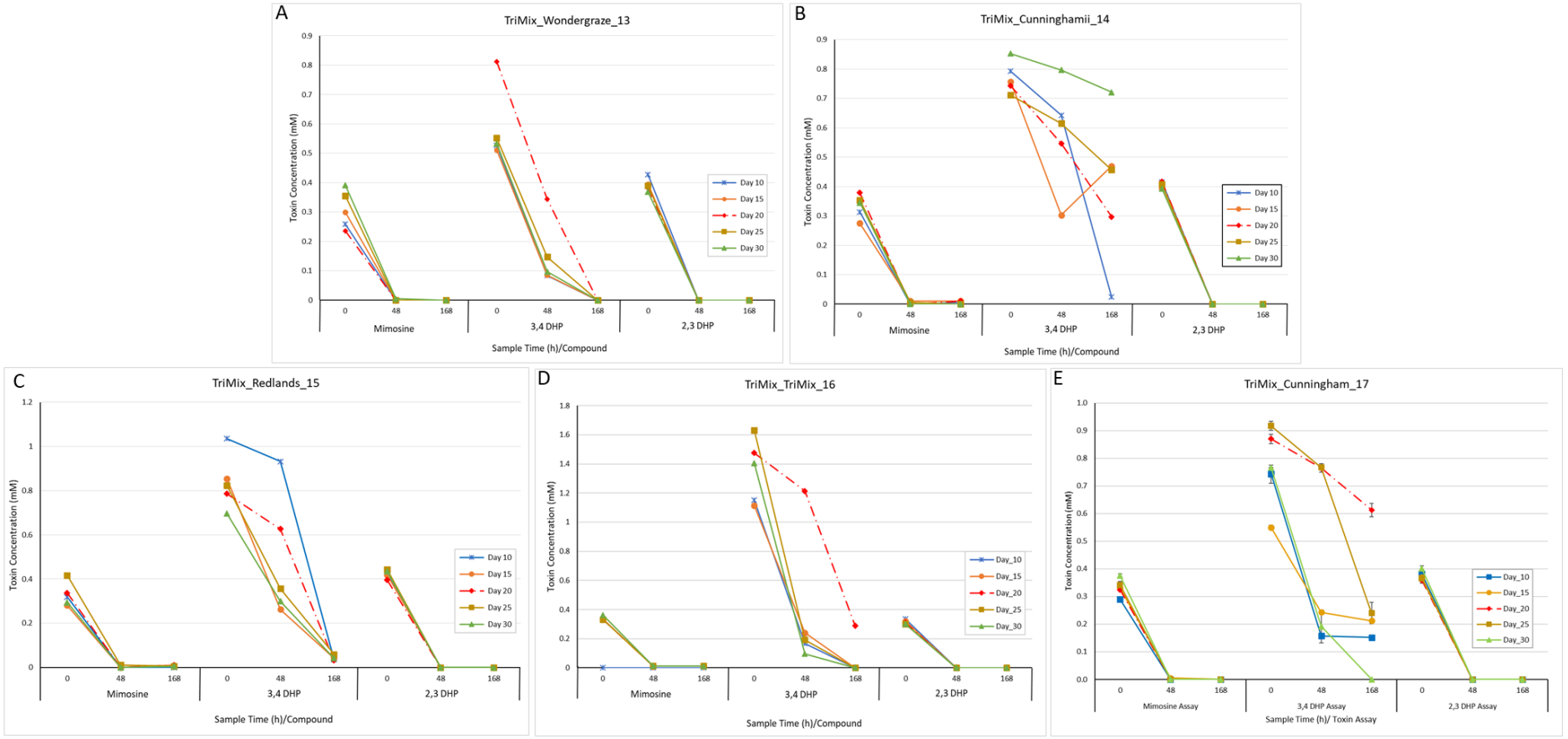
In the TriMix_Cunningham_14 fermentation, the bacterial populations present were able to degrade mimosine and 2,3 DHP completely in toxin degradation assays from day 10 onwards (Fig. 4.9 C). Results of the 3,4 DHP degradation assays for this fermentation were, however, inconsistent. Assays set up on day 10, completely degraded the toxin but the remaining days had incomplete degradation after 168 h incubation and no explanation was found for why the 3,4 DHP level went up in the day 15 assay 168 h sample or for the incomplete degradation in the assays set up days 20, 25 and 30. The fermentation parameters and anaerobic gas appeared to be normal. Samples taken from the 3,4 DHP assay tubes which had be left in the incubator, showed that 73 to 93% of the 3,4 DHP had been degraded.

In the follow-on fermentation TriMix_TriMix_16, degradation assay results were more consistent. Bacterial populations present shown to be able to degrade mimosine, 3,4 DHP and 2,3 DHP completely in all toxin degradation assays, except for the 3,4 DHP in the day 20 degradation assay, which was 80% degraded after 168 h incubation (Fig. 4.9 D).

A repeat TriMix_Cunningham_17 fermentation was undertaken, and the bacterial populations present were able to degrade mimosine and 2,3 DHP completely in all toxin degradation assays from day 10 onwards (Fig. 4.9 E). The 3,4 DHP degradation assays for this fermentation showed degradation of 3,4 DHP reaching complete degradation by day 30. One explanation may be related to potential technical errors, identified after assays were completed. It is not certain why the 3,4 DHP degradation was incomplete as the population of *S. jonesii* was above 10^6 cells/mL throughout the fermentation (Fig. 4. A). One explanation may be related to potential technical errors, identified after assays were completed. There is not a commercial source of 3,4 DHP available, therefore commercially available mimosine is routinely used to synthesise 3,4 DHP ‘in-house’. The toxin can be difficult to dissolve in water and occasionally there may be undissolved crystals, which may have inadvertently transferred into the degradation assay tubes, dissolving over time after the time 0 h sample was taken.

The TriMix_12 fermentation demonstrated that mixed bacterial populations able to digest leaf from three different Leucaena cultivars and detoxify the mimosine, 3,4 DHP and 2,3 DHP could be established in a single fermentation. These TriMix bacterial populations were then able to immediately start increasing, and effectively detoxifying all three toxins, when used in fermentations fed leaf of a single cultivar. It is planned to modify the production of the DAF Leucaena inoculum to the TriMix inoculum for use in cattle grazing different cultivars of Leucaena.

Figure 4.9. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h from toxin degradation assays set up every five days from day 10 to day 30 for A. TriMix Fermentation 13 (Ferm_13); B. TriMix Redlands Fermentation 15 (Ferm 15); C. TriMix Cunningham Fermentation 14 (Ferm_14), D. TriMix TriMix Fermentation 16 (Ferm_ 16) and TriMix Cunningham Fermentation 17.



4.1.2 Microbiome Analysis

Microbiome sequence datasets describing the bacterial and archaeal populations were obtained from CSIRO (received 7/1/2020 and 4/2/2020) for subsamples collected from the eight *in vitro* fermentations of 30-day duration. Microbiome sequence data was also obtained for rumen samples collected throughout the animal trial conducted at CSIRO Lansdown Research Station (received 4/2/2020). Similarly to the microbiome sequence data obtained for the fermenter samples, bacterial and archaeal communities were amplified for the animal trial samples, however the sequence numbers received for the archaeal communities were very low and the animal trial sequence dataset, amplified using the 16S rRNA gene V4 region primers (Kozich et al., 2013) was therefore also used for the analysis of both the bacterial and archaeal populations.

As the DAF component of this project focuses mainly on the efficacy of the inoculum used to break down the toxins present in the different varieties of Leucaena (Cunningham, Redlands and Wondergraze), this report will mainly focus on the microbial communities present in the fermenter and only provides a preliminary analysis of the animal trial microbiome data. It also describes the populations of the bacteria previously shown to detoxify the derivative of bacterial mimosine breakdown (3,4-DHP), which are classified within the phylum Synergistetes, and includes the major species implicated in 3,4-DHP breakdown, *Synergistes jonesii*. Organisms classified within the phylum Synergistetes were detected in the microbiome sequence data from both the fermenter and animal trial samples. The 16S rRNA gene sequences are compared in this report, in order to determine the extent to which the respective fermenter and animal trial *Synergistes* populations are related.

4.1.2.1 Fermenter Bacterial and Archaeal Populations

Sequence data was obtained from samples collected over time from a total of eight, 30-day fermentations. These fermentations were considered to be replicates if they were supplied leaf material from the same Leucaena cultivar throughout the 30-day fermentation period. Two replicate fermentations were supplied with leaf material from the Leucaena cultivar Cunningham, three fermentations were supplied with Wondergraze cultivar leaf material and a further three fermentations were supplied with Redlands cultivar leaf material. The majority of the fermentations were initially inoculated (started) with a microbial inoculum obtained from a previous Cunningham fermentation that had been stored at -20°C. In addition, a single fermentation supplied with the Wondergraze cultivar leaf material was initially inoculated with a microbial inoculum obtained from a previous Wondergraze fermentation and is therefore referred to as a Wondergraze-adapted fermentation. Similarly, a single Redlands fermentation was supplied with Redlands leaf material and was initially inoculated with a microbial inoculum obtained from a previous Redlands fermentation. This fermentation is therefore referred to as a Redlands-adapted fermentation. The fermenter fluid obtained from these two “adapted” fermentations were supplied for use in the animal trial.

Metadata described in the analysis included experimental parameters such as the cultivar of leaf material supplied to the fermenter (either Cunningham, Redlands or Wondergraze) and the fermentation timeline (i.e. day of the fermentation). In addition, groupings from the HPLC data, describing the breakdown of mimosine, 2,3-DHP and 3,4-DHP, were included. Results from a qPCR of fermenter subsamples designed to detect and enumerate *Synergistes jonesii* populations were also included in the microbiome analysis.

Primers designed to amplify the conserved V4 region of the 16S rRNA gene (Kozich et al., 2013) were used to amplify the bacterial populations present in samples obtained from the eight fermentations. These primers can also amplify archaeal populations, therefore analysis of the sequence dataset obtained using these primers also describes the major archaeal populations present in each of the fermentations. In total the single end sequence dataset, following initial quality filtering and length trimming, included 4,446,521 reads, with a mean of 46,317 sequences per sample. The relative abundance of taxonomic groups was ascertained following clustering of the 16S rRNA gene

sequences from each sample into highly related groups termed Features (sequence variants), and a representative sequence for each Feature compared to a database of taxonomically defined microbial groups (SILVA database update 132; Yilmaz et al., 2014). Taxonomy was assigned on the basis of 99% homology and if the Features did not match any of the reference sequences in the database, then they were classified as either unassigned or classified to the highest taxonomic level possible, for example, Kingdom Bacteria. When the negative control samples and low sequence number samples were removed, a total of 1,277 Features were identified across the 88 fermenter fluid samples analysed, representing a total of 1,997,419 16S rRNA gene sequences.

The microbial diversity (alpha diversity) of samples obtained during each fermentation was determined using three diversity measures (Table 4.1). This analysis showed that each fermentation resulted in the successful cultivation of a wide variety of bacteria and archaeal species, irrespective of the cultivar of Leucaena leaf material supplied to the fermentation (Table 4.2). The most significant factor affecting the microbial diversity was the day of fermentation, with all diversity measures examined showing a significant effect ($P < 0.001$) on the bacterial and archaeal populations determined from sequences obtained using the V4 primer set (Table 4.2). Microbial diversity of samples collected throughout the duration of the 30-day fermentation period showed that the growth conditions created in the fermenters enabled sustained cultivation of a highly diverse microbial community (Table 4.1). Statistical analysis of these diversity measures indicated that fermentation time (day of fermentation) had a strong, significant effect on microbial diversity (Table 4.2). For example, microbial diversity was lower during the early days of fermentation than during the final days of the fermentation, when the population diversity was more stable. When factors such as the Leucaena cultivar provided to the fermentation were taken into consideration, the effect on the microbial diversity of the fermenter was not so significant, with only one diversity measure, Faith PD, showing a slightly significant effect ($P = 0.31$). Similarly, when the day of fermentation and cultivar effects were combined, only the Faith PD diversity measure indicated any significant effects. These differences could be attributed to the slightly higher diversity seen in the Wondergraze fermentations.

Table 4.1. Summary of microbial diversity (alpha diversity) measures across the entire sequence dataset for 88 samples from 9 fermentations.

Alpha diversity measure	Mean	Standard deviation
Observed OTUs	148.534	22.71
Shannon	5.360	0.319
Faith PD	15.51	1.418

Table 4.2. Results from a repeated measures residual maximum likelihood (REML) analysis of microbial diversity within each sample (three alpha diversity measures). Statistically significant results (F Pr < 0.05) are highlighted in green.

Fixed effect	Alpha Diversity Measures (F Pr-values)		
	Faith PD	*Shannon*	*Observed species*
Day of Fermentation	<0.001	<0.001	<0.001
Fermentation Cultivar	0.031	0.666	0.57
Day.Fermentation Cultivar	0.008	0.78	0.844

The microbial communities present in the fermentations also showed variation in relative abundance, with certain taxonomic groups present throughout the duration of the fermentation dominating at specific times of the fermentation. This effect was highly repeatable, particularly for the more dominant bacterial taxonomic groups (Fig. 4.10), such as those classified within the orders Bacteroidales, Clostridiales and Selenomonadales. While using the V4 primers for amplification of

archaeal communities was not as sensitive as using the archaeal-specific primers, the archaeal communities identified using the V4 primers and taxonomically classified using the specialist RIM database (version 14_07; Seedorf et al., 2014), also showed distinct changes occurring with time of fermentation (Fig. 4.11). The two major archaeal taxonomic groups identified, representing the genus *Methanosphaera,* and the Family Methanomassiliicoccaceae (currently named in the SILVA database as genus group 9) varied in abundance with time, however the time at which respective populations dominated, did not always show a consistent pattern of fluctuation, with differences occurring between fermentations conducted using the same Leucaena cultivar. Interestingly for most of the fermentations, *Methanosphaera* populations appeared to peak in relative abundance on day 8 of the fermentation.

Overall, at the later days of the fermentation (> 8 days), there was less variation in the types of microbial communities and taxonomic groups present (Fig. 4.12). A previous study, (MLA project B.NBP.072; Davis, 2015), established that as the Leucaena fermentation progresses, the ability of the microbial populations to metabolise mimosine, 3,4-DHP and 2,3-DHP increases. In the current investigation toxin degradation assays and HPLC analysis was conducted for fermenter fluid samples collected on days 10, 15, 20, 25 and 30. Only a small number of samples collected from the Redlands fermentations returned an incomplete result for breakdown of the late-stage toxin by-product, 3,4-DHP. The major microbial populations detected in samples corresponding to the time-points within relatively poor toxin breakdown, did not appear to vary greatly from the other fermentation days when breakdown was complete (Fig. 4.12).

The SPLSDA analysis undertaken enabled the selection of the most predictive or discriminative Features in the dataset (Lê Cao et al., 2011). The SPLSDA plots of all the fermenter samples, showed the distinct effect of fermentation time on microbial community composition, with this effect observed for all of the Leucaena cultivars examined (Fig. 4.13). This analysis also showed that there was less variation occurring between the bacterial and archaeal communities present at the later days of each fermentation. This was indicated by the spatial convergence of sample populations represented in the SPLSDA plots (Fig. 4.13). This analysis was also used as the basis for determining which microbial communities were contributing to the differences observed between samples, such as those which changed in relative abundance according to the time (day) of fermentation (Fig. 4.14). The organisms contributing to the difference occurring between days of the fermentations include those which declined in abundance over time e.g. populations classified within the families Veillonellaceae, Bacteroidaceae, Anaeroplasmataceae and some genera classified within the family Lachnospiraceae. Families which increased in relative abundance with time include Ruminococcaceae, Spirochaetaceae, Rikenellaceae, Prevotellaceae, Mollicutes and Synergistaceae (Fig. 4.14 and Fig.4.15).

Given that the microbial communities were more stable and consistent at the later days of all eight fermentations, the “core” microbial communities could be determined. Core communities were defined as those which were present in 100% of fermenter fluid samples collected at ≥ 10 days (days 10, 15, 20, 25 and 30) of all fermentations and represented microbial populations of relatively high abundance (Maximum 81,169 reads per Feature and Minimum 115 reads per Feature; 5, 891 ± 12,320 mean reads per Feature ± standard deviation). Later day core communities are listed in Appendix 12.4 (Table 12.4), with a total of 41 microbial Features identified as being present in all eight fermentations, with all of these communities being present at high numbers in the sequence dataset.

Core communities were also determined for replicate fermentations maintained on the same Leucaena cultivar. When core fermenter communities corresponding to each of the three Leucaena cultivars were compared (Fig. 4.16), a high proportion (70.7%) of the core community was present in all of the fermentations and included taxonomic groups usually associated with rumen fibre breakdown such as those classified in the phyla Bacteroidetes and Firmicutes, with highly abundant genera including *Prevotella*, the Rikenellaceae RC9 gut group, *Anaerovibrio*, *Butyrivibrio* and

Desulfovibrio. Core communities found in all cultivars included representatives of the phylum Synergistetes, notably the genus *Pyramidobacter*. The methanogenic archaeal genus *Methanosphaera* was the only core archaeal community found to be present in the later days of fermentations with the three Leucaena cultivars. Several unique, cultivar-specific core microbial communities were identified (Table 4.3), however these communities represented a relatively small proportion (15.4%) of the overall number of core communities identified. Interestingly, the archaeal Family Methanomethylophilaceae, was identified as a core microbial community in only the Cunningham and Wondergraze fermentations. The bacterial species known to be involved in DHP breakdown, *S. jonesii,* was found in a large proportion of fermenter fluid samples collected on the later days (80% of fermenter fluid samples collected from≥ day 10 of the Cunningham; Redlands and Wondergraze fermentations), and although often detected, this species was not designated as a "core" microbial community.

Similarly, when all the identified fermentation cultivar-specific microbial communities (> 5 sequences per Feature) from the later days (≥ day 10) were examined, a high proportion of bacteria and archaea were found to be present in eight fermentations (127 Features, 73.4% of identified Features). Bacterial communities found in all fermentations included representatives of organisms belonging to the phylum Synergistetes, such as *S. jonesii*. Archaeal communities found in all samples included those belonging to the genera *Methanosphaera*, *Pyramidobacter*, an uncultured Methanomethylophilaceae and *Methanobrevibacter* (Table 4.3). Interestingly, the methanogen species which usually dominates rumen microbial communities, *Methanobrevibacter ruminantium*, was only present in the fermenter fluid samples collected on the later days of replicate Wondergraze fermentations.

The analysis of all the microbiome sequence datasets obtained, showed that the bacterial and archaeal communities of eight fermentations, irrespective of the Leucaena cultivar supplied, fluctuated in diversity and community structure according to the time (day) of fermentation. The first 2 to 8 days of the fermentations showed the most variation, with later days (≥ day 10) of the 30-day fermentation being more stable in regards to diversity and microbial community structure. This indicates that the microbial communities of the fermenter may undergo competition and selection for those microbial populations which can best survive in the physical and chemical conditions of the fermentation vessel. Given that all fermentations are first supplied with the same rumen fluid-based growth medium, the chemical and nutrient conditions within the fermentations are determined by the leaf material supplied and the microbial communities contained within the fermenter, including the waste products of microbial fermentation.

These time-related changes in microbial diversity and community structure were more apparent in the bacterial than the archaeal communities of the fermenter. While the Leucaena cultivar provided to respective fermentations did encourage the growth of some cultivar-specific bacterial populations, many bacterial genera were found in all fermentations, particularly during the later days of the fermentation (≥ 10 days). This indicates that irrespective of the Leucaena cultivar supplied to the fermentation, a large "core" microbial community will develop to utilise the plant material provided. This "core" microbial community includes genera known to degrade both simple and complex plant sugars (e.g. the family Lachnospiraceae including the genera *Clostridium*, *Eubacterium* and *Ruminococcus*) and metabolically diverse populations such as those known to degrade plant proteins (e.g. the family Prevotellaceae). The latter populations may usually be found in higher concentrations within fermenters supplied with Leucaena plant material than would normally be expected in cattle rumen samples, as Leucaena plant material contains relatively high concentrations of protein (Albores-Moreno et al., 2019). Further analysis of other chemical compounds contained in the different cultivars of Leucaena (e.g. plant tannins) may indicate whether the cultivar-dependant microbial populations identified in this study were being selected and their growth encouraged due to their tolerance or ability to break down cultivar-specific plant compounds.

Figure 4.10. Changes in the bacterial and archaeal populations of all replicate Leucaena cultivar fermentations, ordered according to time (day of fermentation). Populations are classified to the taxonomic level of order, using the SILVA database (132 update).

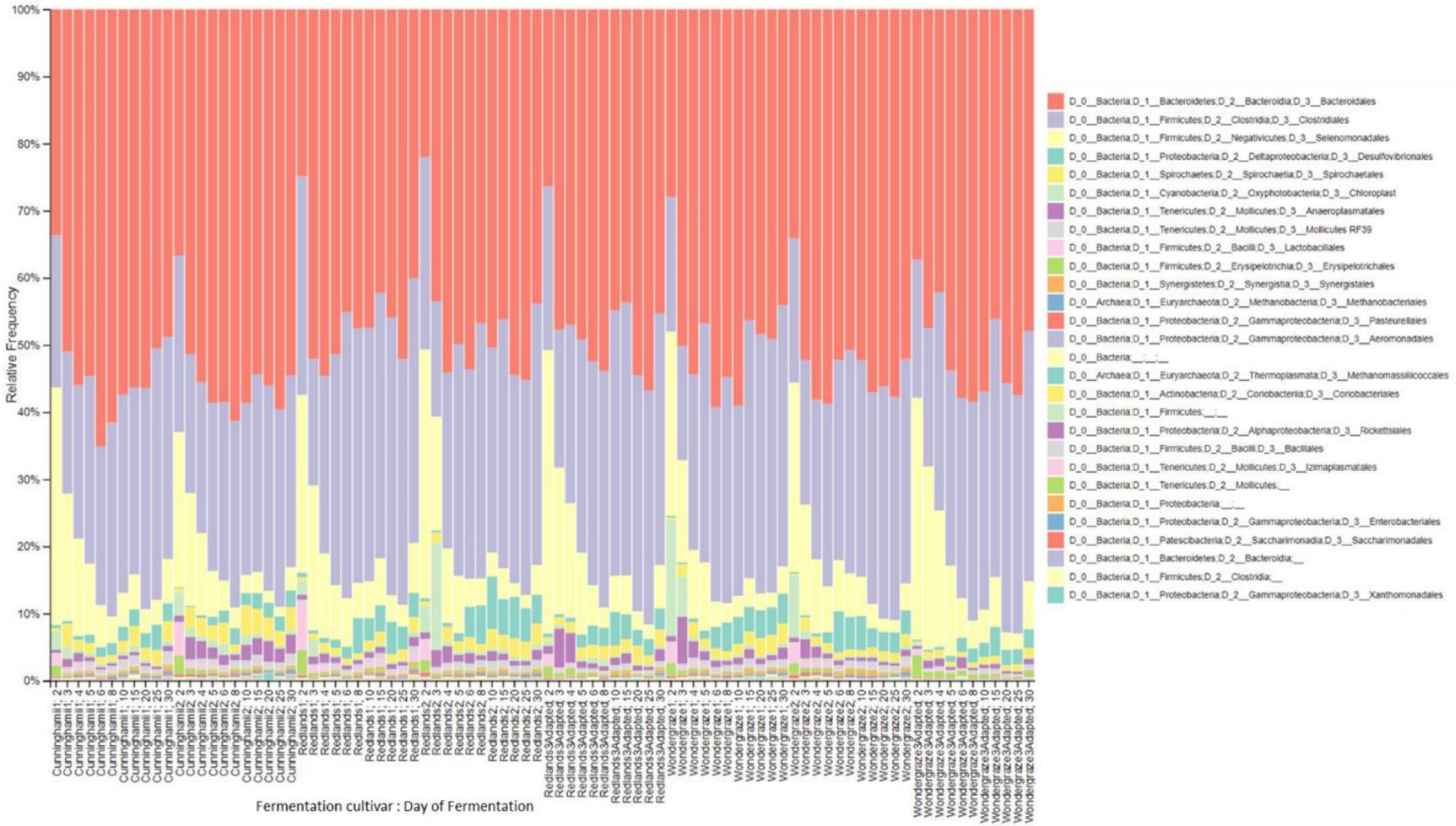


Figure 4.11. Changes in the archaeal populations of all replicate Leucaena cultivar fermentations, ordered according to the time (day of fermentation). Archaeal populations determined by V4 primer dataset and classified to the taxonomic level of species, using the RIM database (version 14_07).

Figure 4.12. Changes in the bacterial and archaeal populations of all replicate Leucaena cultivar fermentations at the later days of the fermentation (≥ day 10), ordered according to the time of fermentation (day), with the HPLC determination of 3,4-DHP breakdown noted. Taxonomy reported at genus level classification (SILVA 132 database).

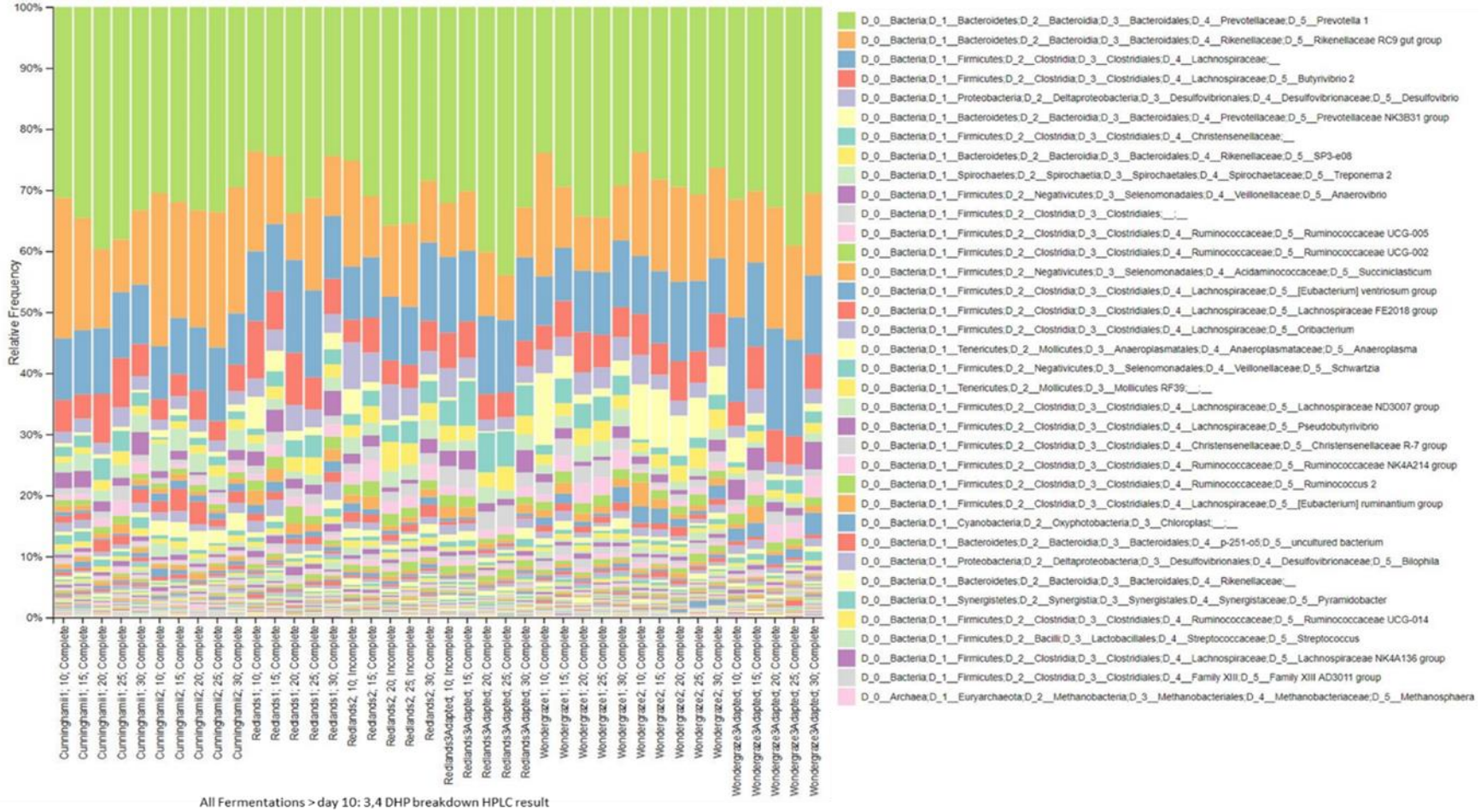


Figure 4.13. Partial Least Squares Discriminant Analysis (sparse) (sPLSDA, Components 1 vs 2 and Components 1 vs 3) of bacterial and archaeal populations in replicated fermentations indicating separation of sample populations of the basis of the time (day) of the fermentation. The Leucaena cultivars provided to respective fermentations are depicted using symbol shape.

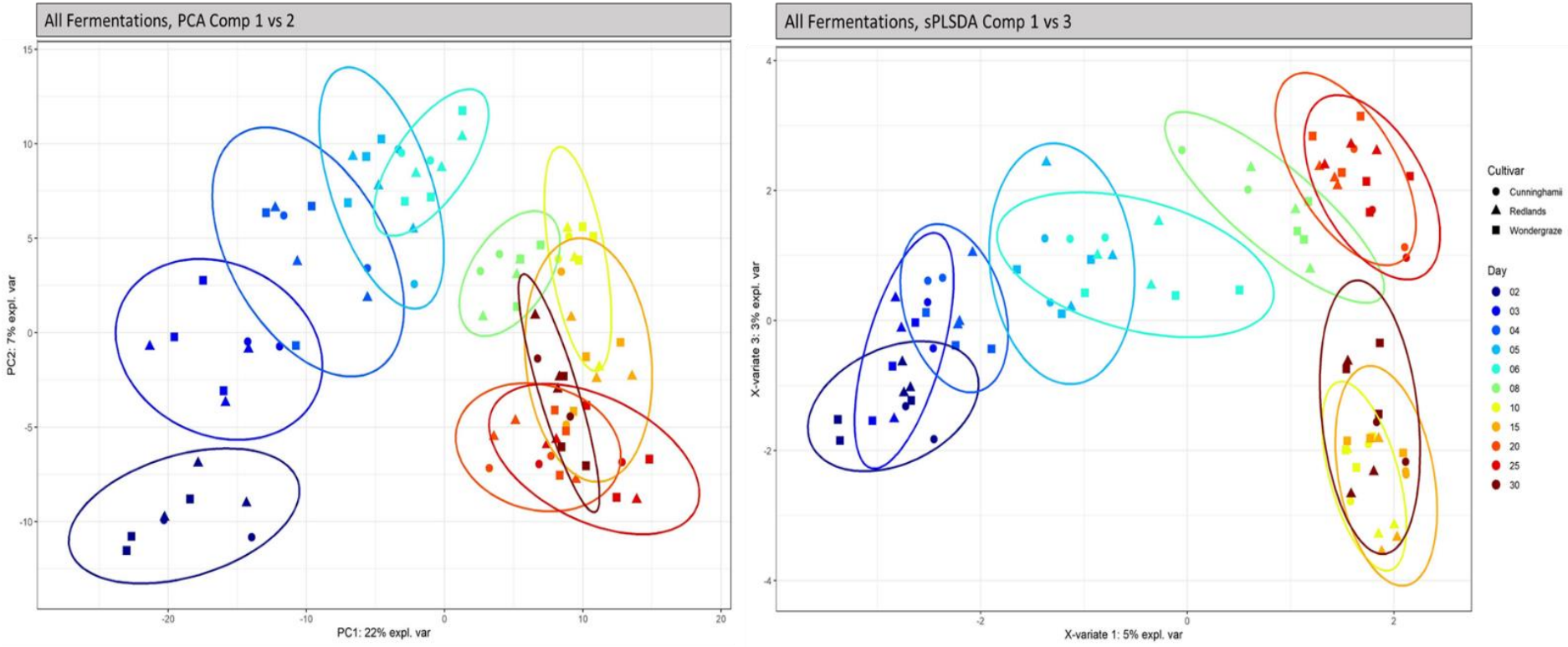


Figure 4.14. Contribution plots generated from the Partial Least Squares Discriminant Analysis (sparse) components 1 to 3, showing which microbial families contributed to the differences occurring throughout the duration (days) of eight Leucaena Fermentations. Microbial families are represented on bar plots, with the negative or positive correlation indicated by the direction of the bars in relation to the scale below each plot. Bars are coloured according to the day on which they contributed the most effect within the SPLSDA.

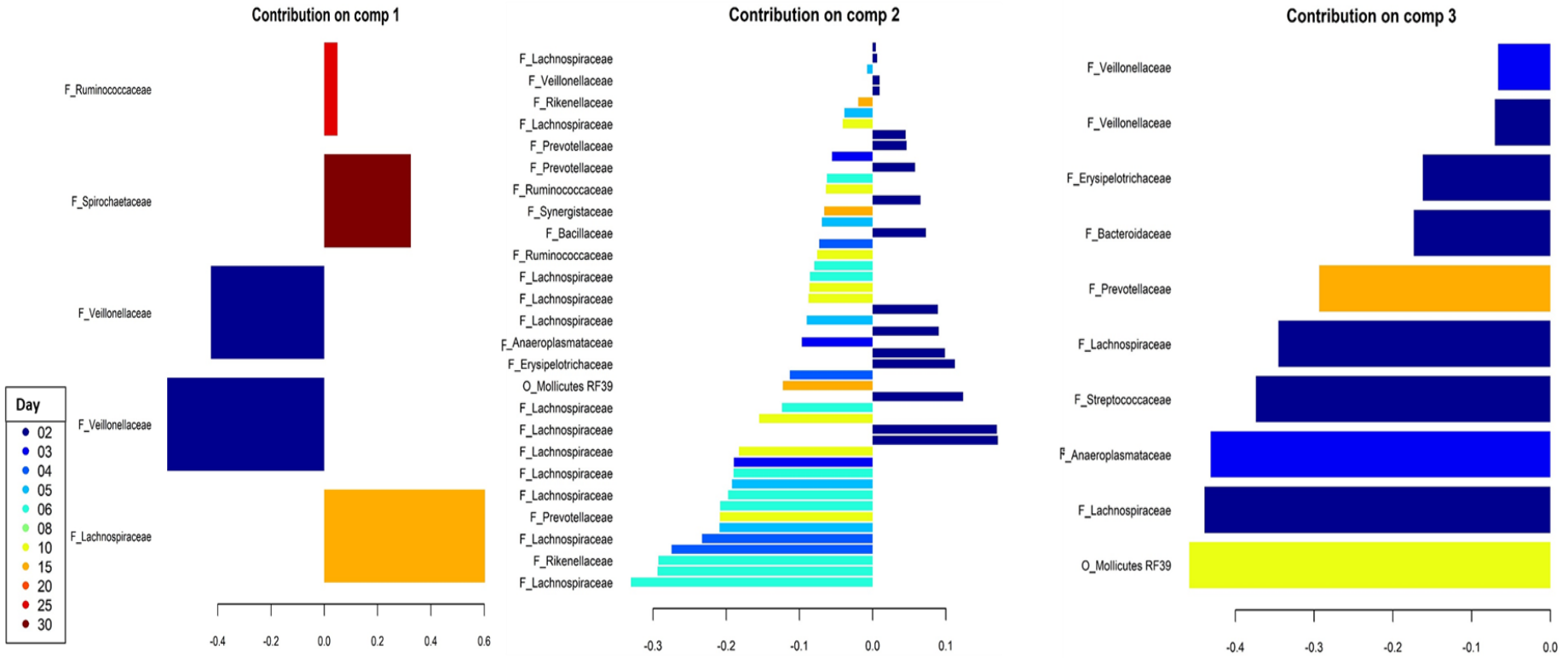


Figure 4.15. Clustered image map of all families identified as contributing to differences occurring between sample days of eight Leucaena Fermentations. Differences were determined from the Partial Least Squares (sparse) matrix of microbial families also used to generate the figures above. The matrix is graphically represented with each entry of the matrix coloured on the basis of contribution (see colour key). The rows and columns are ordered according to a hierarchical clustering. Dendrograms resulting from the clustering are on left and to the top of the image, with a bar indicating the day included.

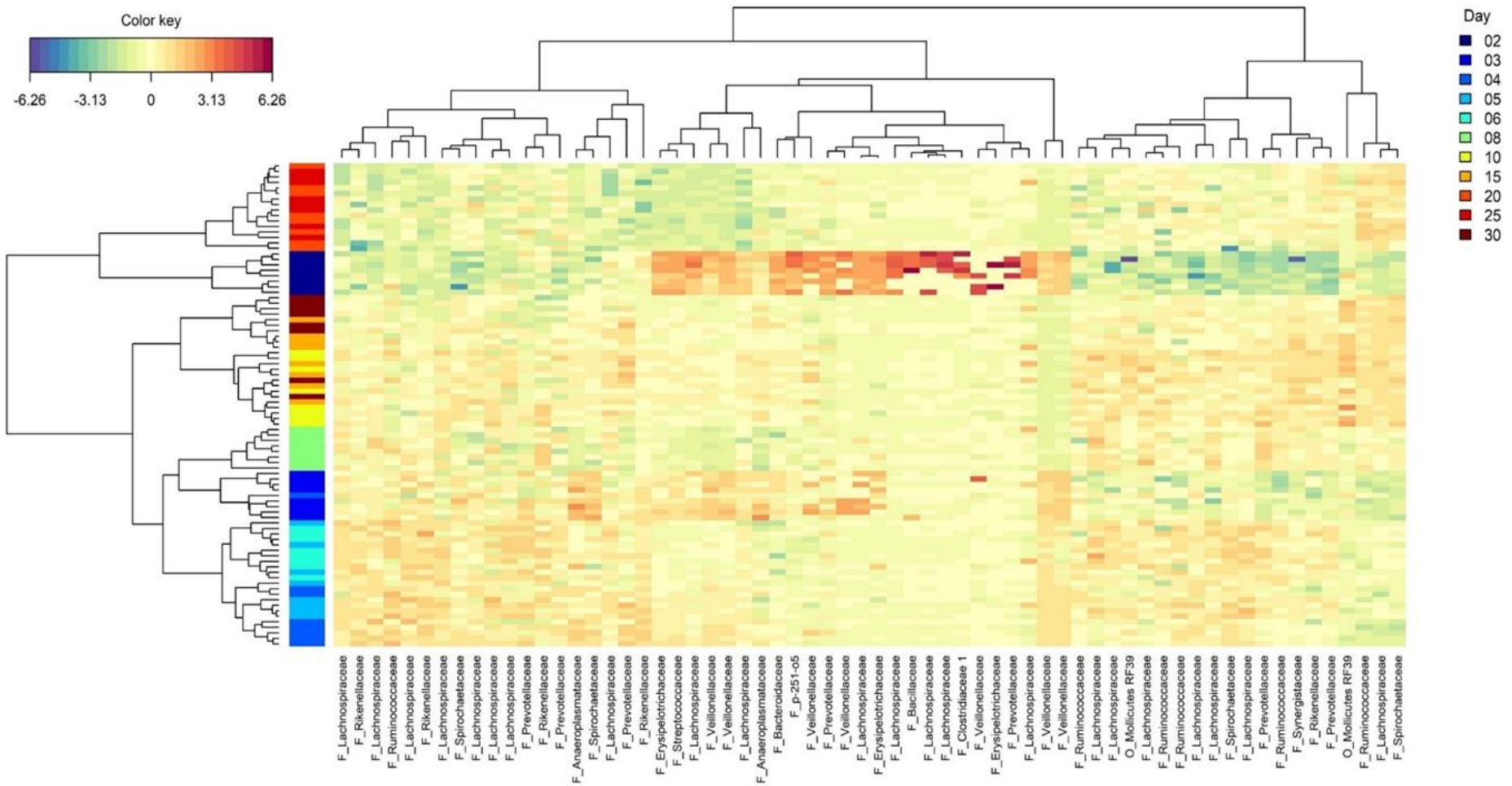


Figure 4.16. Comparison of bacterial and archaeal populations (Features classified at species level) present at the later days of fermentations (≥ 10 days), according to the Leucaena cultivar provided to the fermentation, visualised in Venn diagrams. The proportions of bacterial and archaeal communities that were either shared or unique, are presented as the actual number of Features and as a percentage of the overall population. Bacterial and archaeal populations were designated as core microbial populations if they were present in all the samples collected on days 10, 15, 20, 25 and 30, from replicate fermentations maintained on each Leucaena cultivar (either two Cunningham fermentations, three Redlands fermentations or three Wondergraze fermentations).

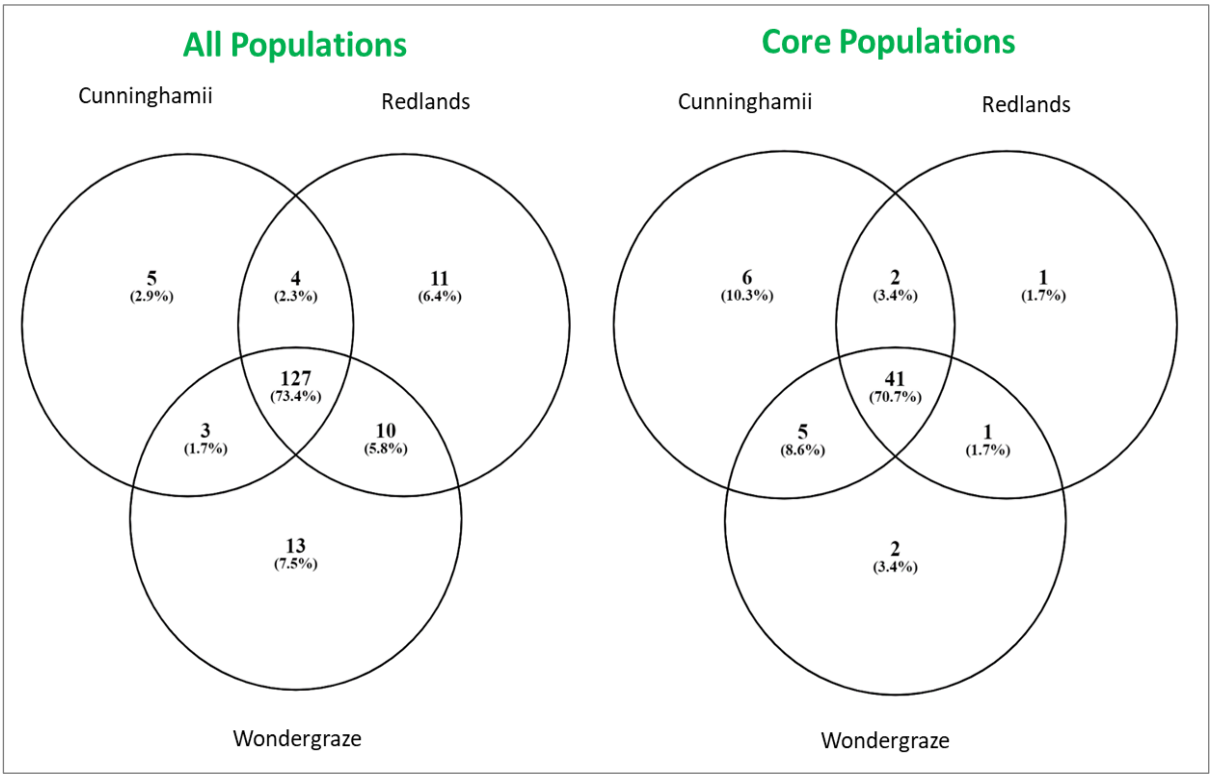


Table 4.3. Listing of core microbial communities from the later fermentation days (≥ 10 days), found to be cultivar-dependant or unique. Features classified according to SILVA database (version 132) taxonomic classification levels of D_0, Domain; D_1, Kingdom; D_2, Phylum; D_3, Class; D_4, Order; D_5, Family; D_6, Genus; D_7, Species.

Unique Features, two Cunningham Fermentations
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;D_5__Family XIII AD3011 group;D_6__bacterium AD3011
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Rikenellaceae;D_5__Rikenellaceae RC9 gut group;D_6__bacterium enrichment culture clone RB2a
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pasteurellales;D_4__Pasteurellaceae;D_5__Basfia
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Bacteroidaceae;D_5__Bacteroides
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__*Eubacterium coprostanoligenes* group
D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Catenisphaera
Unique Feature, three Redlands Fermentations
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;D_5__Family XIII AD3011 group
Unique Features, three Wondergraze Fermentations
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Rikenellaceae;D_5__Rikenellaceae RC9 gut group;D_6__uncultured rumen bacterium
D_0__Bacteria;D_1__Spirochaetes;D_2__Spirochaetia;D_3__Spirochaetales;D_4__Spirochaetaceae;D_5__Treponema 2;D_6__bacterium MD2012
Common Features, Cunningham and Wondergraze Fermentations
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae ND3007 group
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae NK4A136 group;D_6__bacterium XPB1013
D_0__Archaea;D_1__Euryarchaeota;D_2__Thermoplasmata;D_3__Methanomassiliicoccales;D_4__Methanomethylophilaceae
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-010
D_0__Bacteria;D_1__Spirochaetes;D_2__Spirochaetia;D_3__Spirochaetales;D_4__Spirochaetaceae;D_5__Treponema 2;D_6__bacterium WCE3006
Common Features, Cunningham and Redlands Fermentations
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Christensenellaceae;D_5__Christensenellaceae R-7 group
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Eubacterium] hallii group;D_6__uncultured Lachnospiraceae bacterium
Common Feature, Redlands and Wondergraze Fermentations
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcus 1;D_6__*Ruminococcus flavefaciens*

Archaeal population of the fermentations were further examined using a sequence dataset generated using archaea-specific primers and resulted in sufficient sequence read numbers to enable determination of the archaeal community structure within the fermentations. For the 88 fermenter fluid samples examined, 75 Features were identified representing 2,676,236 16s rRNA gene sequences, following all quality filtering steps and removal of any erroneous bacterial sequences. Even with the use of archaea-specific primers, the number of archaeal Features identified within the sequence dataset was low (Table 4.4). For eight fermentations, the archaeal communities were dominated by two archaeal populations, taxonomically assigned to the genus *Methanosphaera,* and family Methanomassiliicoccaceae (group 9). While other species of *Methanobrevibacter* were observed, including *M. gottschalkii, M. arboriphilus, M. ruminantium, M. smithii* and *M. boviskoreani*, these were usually found at very low levels (less than 0.5% relative abundance) (Fig. 4.17).

As established with the V4 primers, archaeal populations were less stable during the later days of each fermentation (Fig. 4.18), with the proportions of the two dominant microbial populations fluctuating throughout the duration of each of the fermentations. For some fermentations, for example, two of the Redlands fermentations, the archaeal genus *Methanosphaera* dominated (> 69.9% relative abundance from days 3-30). The effect of the Cunningham and Wondergraze cultivars on archaeal populations of replicate fermentations was variable. For example, in Cunningham fermentation 1, *Methanospheara* populations accounted for up to 98.7% of total archaeal populations whereas in Cunningham fermentation 2, the family Methanomassiliicoccaceae was generally more abundant (relative abundance up to 77.4%). Fermentations fed the Wondergraze cultivar were also very variable, with fermenter fluid samples from the first Wondergraze fermentation showing *Methanosphaera* abundance up to 87%. The second Wondergraze fermentation however, was completely dominated by populations of *Methanosphaera* (> 99.04% after day 3). In contrast, the fermentation started with a microbial inoculum obtained from a previous Wondergraze fermentation, had very high levels of the family Methanomassiliicoccaceae (up to 99.7% relative abundance).

Determination of archaeal population diversity within fermenter fluid samples (alpha diversity measures, Table 4.4) indicated that although there was some significant effect (F Pr < 0.05) associated with the time (day) of the fermentations, this effect was not seen for all the alpha diversity measures determined (Shannon and Observed species only, Table 4.5). The effect of the Leucaena cultivar provided to the fermentations was not strongly supported by the repeated measures residual maximum likelihood (REML) analysis, with only one of the diversity measures, which takes into account the phylogenetic diversity of samples (Faith PD), indicating a significant effect of cultivar. When both the effects of day and cultivar were examined together, there were no significant effects seen for any of the diversity measures.

The sPLSDA helped to provide a more in-depth picture of how the archaeal populations of the fermenter varied with time (Fig. 4.19). This analysis confirmed that although archaeal population diversity showed some significant changes in diversity with time of fermentation, overall, the fermenter archaeal populations did not fluctuate to the same extent as the bacterial populations, with far less spatial separation of samples visualised in the sPLSDA plots (Fig. 4.19). The only exception to this was that the fermenter fluid collected on the final days of the fermentation (day 30) appeared to have different microbial populations to those found on earlier days of the fermentation, particularly to those present on the initial days of the fermentation (for example, day 2).

The archaeal populations contributing to the differences seen between the early and late days of the fermentations included low abundance populations classified within the genus *Methanobrevibacter*, and additional, lower abundance species of *Methanosphaera*, which were found to be present in samples collected on the late days of the fermentations (e.g. days 25 and 30) (Fig. 4.20). The dominance of *Methanosphaera* populations during the mid-stages of the fermentations (e.g. days 8

and 10), also contributed to the spatial separation of overall archaeal populations on the basis of time in the sPLSDA (Fig. 4.21).

These results indicated that the archaeal populations present in the fermentations were not as diverse as those normally seen in the rumen, with all fermentations being dominated by two archaeal populations classified as the genus *Methanosphaera* and family Methanomassiliicoccaceae (group 9). While other genera, such as *Methanobrevibacter* were detected, for all the Leucaena fermentations, those were present at very low concentrations. Interestingly, both of the archaeal populations which survived and proliferated throughout the duration of the Leucaena fermentations, are methanogens which are known to utilise hydrogen to reduce methanol to methane. In contrast, the methanogens which are often highly abundant in the rumen of cattle are those classified within the genus *Methanobrevibacter*, which use formate to reduce carbon dioxide in the presence of hydrogen, and generally produce higher concentrations of methane than the methanogens which utilise methanol.

Table 4.4. Summary of microbial diversity (alpha diversity) measures across the entire archaeal sequence dataset of 84 samples from 9 fermentations.

Alpha diversity measure	Mean	Standard deviation
Observed OTUs	7.69	1.79
Shannon	1.24	0.63
Faith PD	1.35	0.02

Table 4.5. Results from a repeated measures residual maximum likelihood (REML) analysis of archaeal population diversity within each sample (three alpha diversity measures). Statistically significant results (F Pr < 0.05) are highlighted in green.

Fixed effect	Alpha Diversity Measures (F Pr-values)		
	Faith PD	*Shannon*	*Observed species*
Day of Fermentation	0.773	0.004	0.002
Fermentation Cultivar	0.011	0.187	0.3
Day.Fermentation Cultivar	0.902	0.425	0.29

Figure 4.17. Changes in the archaeal populations of all replicate Leucaena cultivar fermentations, ordered according to time (day of fermentation). Populations are classified to the taxonomic level of species, using the RIM database (version 14_07).

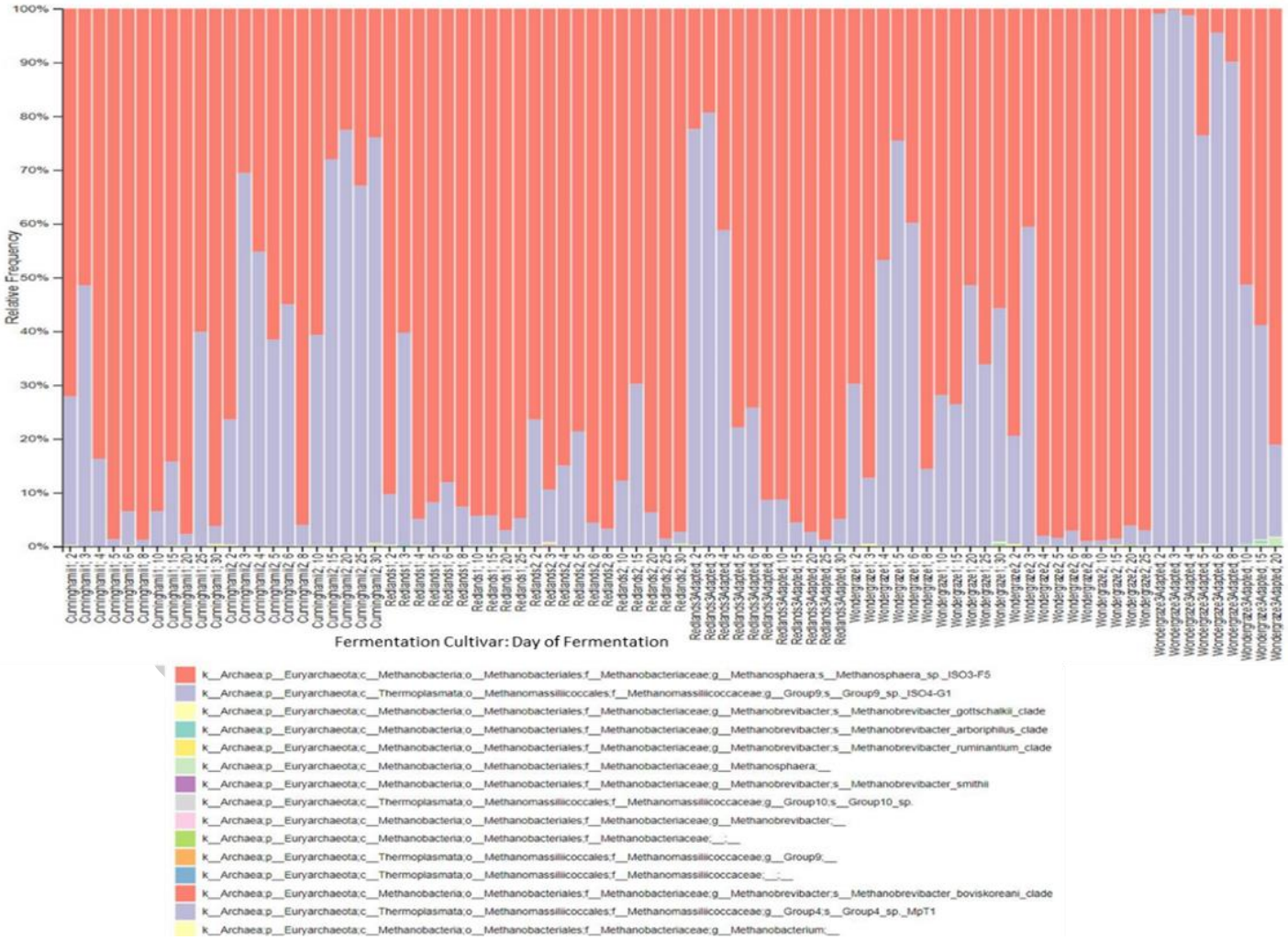


Figure 4.18. Changes in the archaeal populations of all replicate Leucaena cultivar fermentations at the later days of the fermentation only (≥ fermentation day 8), ordered according to the time of fermentation (day). Archaeal populations determined by specific archaeal primers and classified to the taxonomic level of species, using the RIM database (Version 14_07).

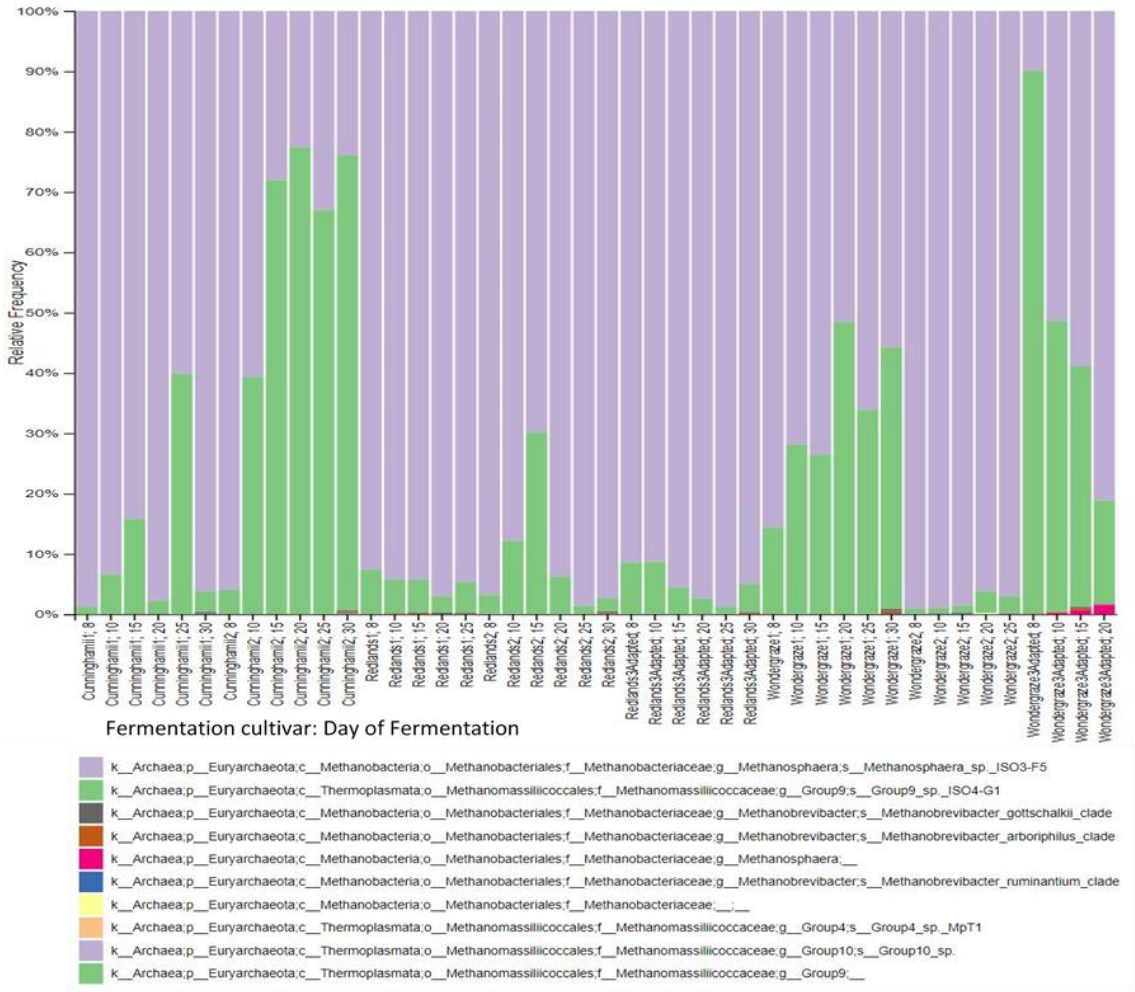


Figure 4.19. Partial Least Squares Discriminant Analysis (sparse) (sPLSDA, Components 1 vs 2 and Components 1 vs 3) of archaeal populations in replicated fermentations. The Leucaena cultivars provided to respective fermentations are depicted using symbol shapes.

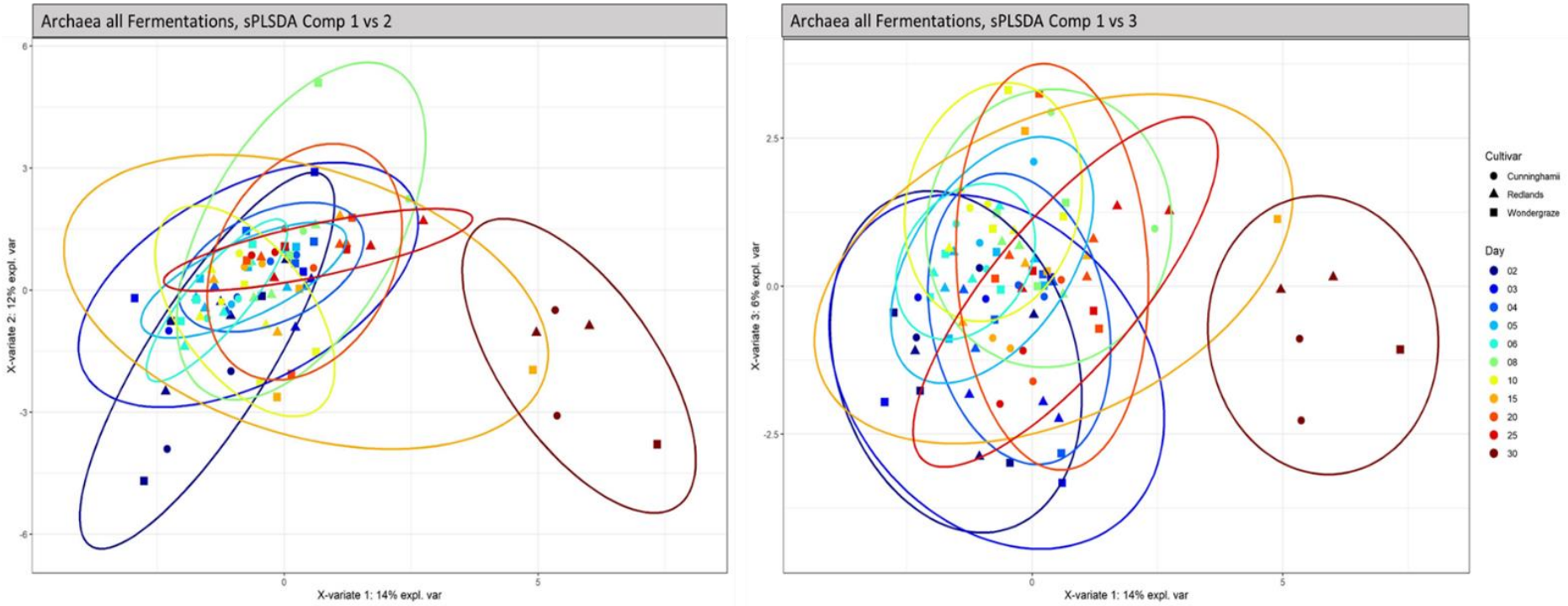


Figure 4.20. Contribution plots generated from the Partial Least Squares Discriminant Analysis (sparse) components 1 to 3, showing which archaeal families contributed to the differences observed throughout the duration (days) of eight Leucaena fermentations. Microbial families are represented on bar plots, with the negative or positive correlation indicated by the direction of the bars in relation to the scale below each plot. Bars are also coloured according to the day on which they contributed the most effect. The name f_Methanomassilic is a truncation of the full family name, Methanomassiliicoccaceae.

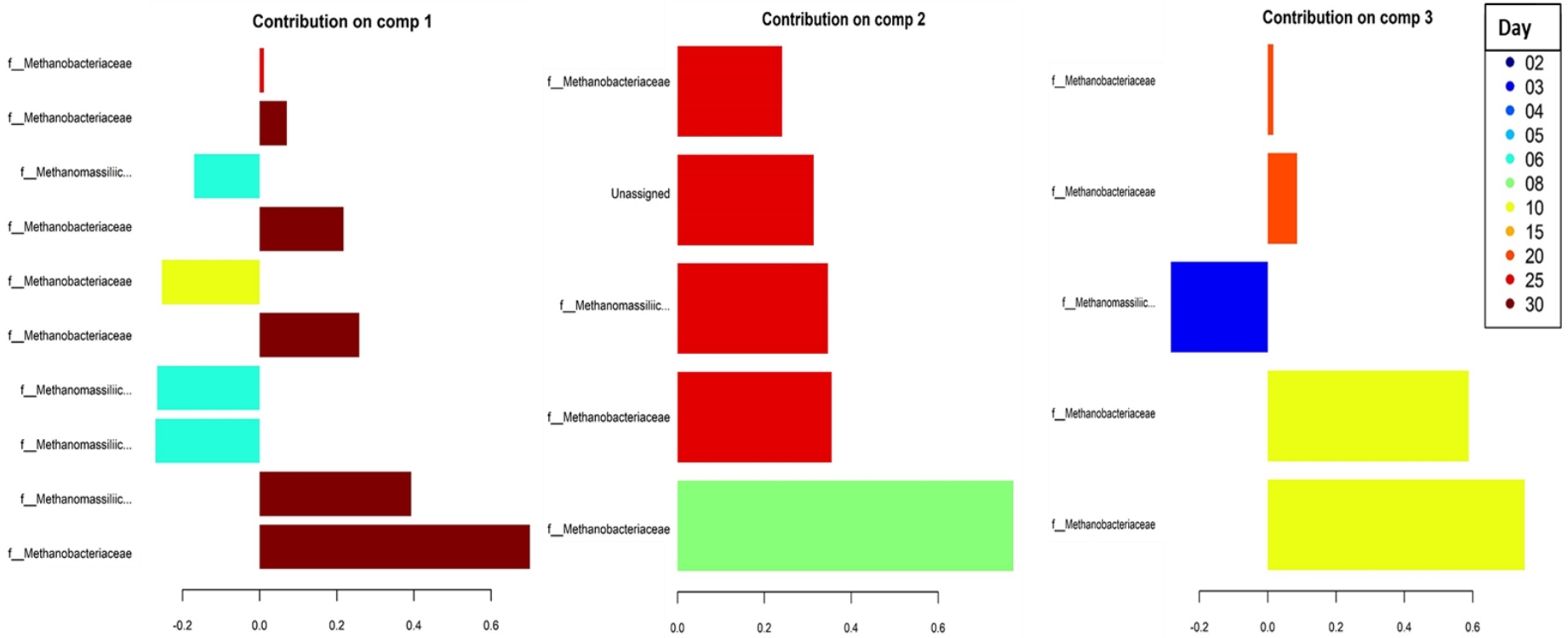
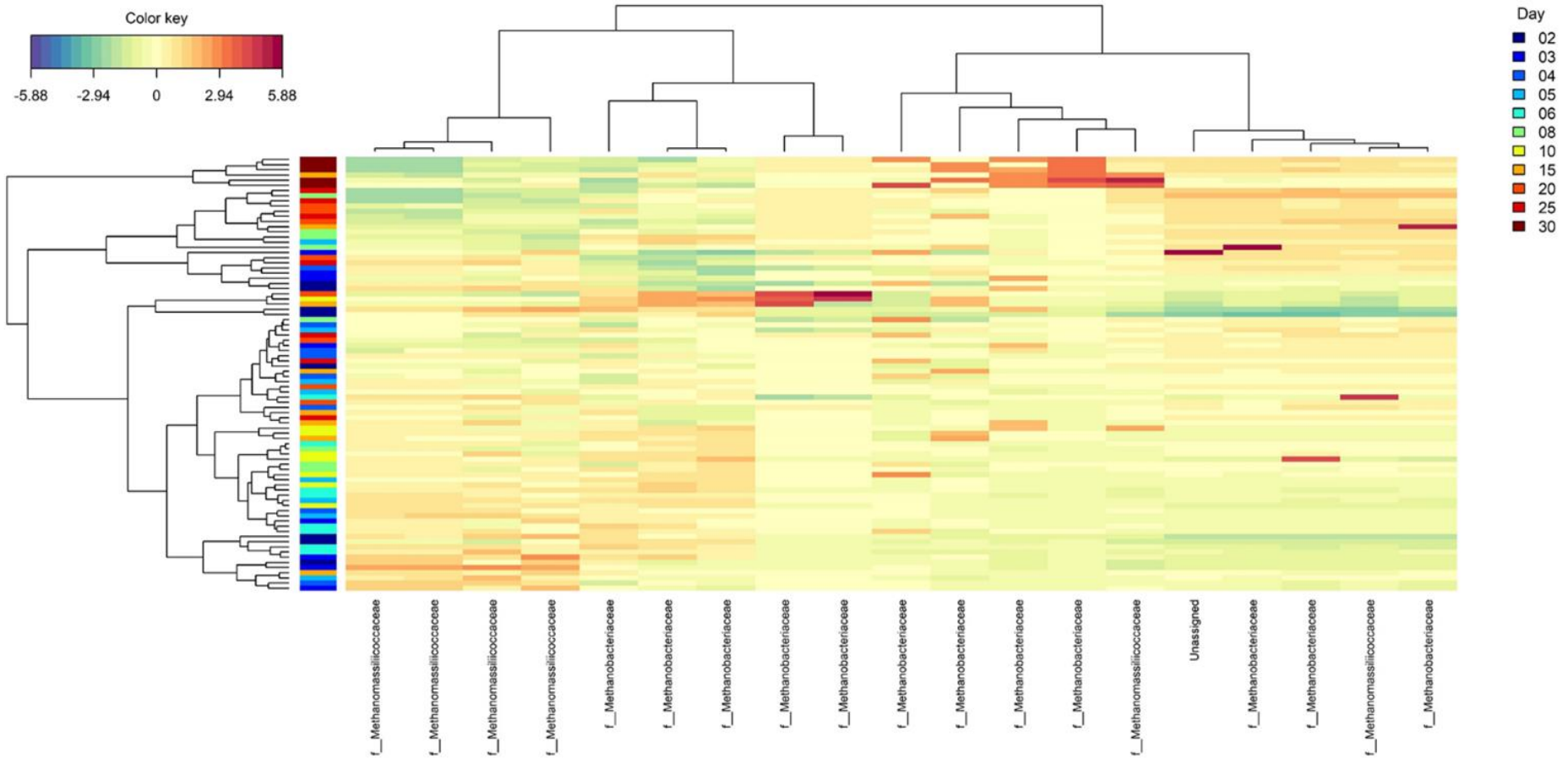


Figure 4.21. Clustered image map of all archaeal families identified as contributing to differences occurring between sample days of eight Leucaena fermentations. Differences were determined from the Partial Least Squares (sparse) matrix of microbial families also used to generate the SPLSDA plots above. The matrix is graphically represented with each entry of the matrix coloured on the basis of contribution (see colour key). The rows and columns are ordered according to a hierarchical clustering. Dendrograms resulting from the clustering are on left and to the top of the image, with a bar indicating the day included.



4.1.2.2 Animal Trial Bacterial and Archaeal populations

Rumen samples were collected from 16 animals on nine different occasions throughout the duration of the animal trial. The dataset describing both the bacterial and archaeal populations based on amplification of the V4 region of the 16S rRNA gene encompassed a total 3,877,567 sequences (mean of 27,115 sequences per rumen fluid sample) representing 13,811 Features, following all quality filtering steps, removal of Features represented by < 5 reads and removal of all sequencing controls. The sequence dataset obtained using archaea-specific primers following all quality filtering (not including the filter to remove Features represented by <5 reads) generated only 37, 036 sequences representing 157 Features, with a mean of 250 sequences per rumen fluid sample, therefore analysis of this relatively small dataset is not described in this report.

The microbiome sequence dataset of the rumen fluid samples indicated that, overall, the rumen fluid had a higher microbial diversity than that found in the Leucaena fermentations (Table 4.1 and Table 4.6)**.** For example, the mean Observed OTUs for the fermentations was 148.53 ± 22.71 (mean ± SD) and the mean Observed OTUs of rumen fluid samples, was approximately 1.5 times higher at 224 ± 86.15 (mean ± SD). The sequence depth obtained for the 88 fermenter samples however, was greater than that obtained for the animal trial rumen samples, therefore the microbial populations of the fermenter could be more comprehensively described. These differences in sequencing depth may also have impacted on the ability to detect specific minor microbial populations of interest, such as *S. jonesii* and those classified within the phylum Synergistetes (further described in Section 4.1.2.3 below).

Table 4.6. Summary of microbial diversity (alpha diversity) measures across the entire archaeal sequence dataset of 189 rumen fluid samples.

Alpha diversity measure	Mean	Standard deviation
Observed OTUs	224	86.15456
Shannon	6.693485	0.718572
Faith PD	27.3931	6.550577

Analysis of the animal trial sequence dataset (Fig. 4.22) showed that there were few differences between the microbial populations during the feeding trial itself. The major differences in microbial populations appeared at the start of the trial, occurring between the initial baseline sampling and the first dietary period. The drenching of animals with cultivar-specific inoculums, collected and cryopreserved from fermentations supplied with either Redlands or Wondergraze cultivar leaf material, appeared to have little effect on the microbial populations of animals supplied either of these cultivars in their diet.

Other than the initial changes in microbial populations which occurred as cattle transitioned into the feeding trial, rumen samples collected during sample collection period three appeared to differ from those collected at the end of this dietary testing stage (sample collection period 4) (Fig. 4.22 and Fig. 4.23). The reason for these changes remains unexplained. It can be presumed that these changes would be unrelated to any microbial changes resulting from cultivar-specific microbial inoculation, given that these changes were seen for all animals at this time, including animals provided with the control diet. When the pre- and post-inoculation sample collections were removed from the analysis, the same effects were observed (Fig. 4.24 and Fig. 4.25), with differences between the baseline sampling and all dietary periods, and differences occurring at the third sample collection period. These changes were largely driven by populations such as those classified within many families such as Bacteroidales and Ruminococcaceae, present in the baseline rumen samples and populations of the family Prevotellaceae present in samples from period three.

The animal trial conducted by CSIRO was designed to test the effects of feeding cattle three different concentrations (18, 36 and 48% Leucaena) of two different Leucaena cultivars (Redlands and

Wondergraze) on enteric methane production and rumen microbial populations including methanogens. The microbial inoculum collected and cryopreserved from the Wondergraze-adapted fermentation and the Redlands-adapted fermentations were administered (via drench gun) to the cattle prior to their transition to diet treatment groups for each respective Leucaena cultivar. On the day of inoculation, a rumen sample was taken immediately prior to drenching with inoculum and three hours after drenching.

The results reported represent an overview of the sequence dataset obtained for the animal trial however this clearly showed that the main difference in microbial populations occurred following the transition of animals into the trial (i.e. from the baseline sampling to the first pre-inoculation sampling). There were also some changes occurring at the third dietary sampling period. These changes did not appear to be related to any effects which may have occurred as a result of administering the Leucaena cultivar-specific mixed microbial inoculum. Indeed, sPLSDA plots showed that there was very little difference in the rumen microbiome of cattle pre- and post-inoculation. In addition, as the animal trial samples (148 samples in total) were all sequenced in one Illumina lane, the sequence depth per sample was relatively low. While the sequencing depth was sufficient for determination of the major, most abundant rumen microbial communities, minor microbial populations such as those classified in the phylum Synergistetes were detected in very low numbers in a low number of rumen samples. For example, *S. jonesii* was detected in microbiome sequence data from one control animal at one collection time-point. In this regard, the microbiome sequence data obtained for the animal trial was not considered to be adequate for determining whether the microbes contained in the cultivar-specific mixed microbial inoculums were surviving and establishing within the rumen of cattle being fed either the Redlands or Wondergraze cultivars.

Figure 4.22. Partial Least Squares Discriminant Analysis (sparse) (sPLSDA, Components 1 vs 2 and Components 1 vs 3) of bacterial and archaeal populations from all rumen samples from the animal trial. Labelling of samples is on the basis of animal number_Diet1.Diet2.%Leucaena. The nine sample collection sessions are coloured accordingly (1 BL= Baseline; 2 Pre1= Pre-inoculation sampling 1; 3 Post2= 3h post-inoculation sampling 1; 4 P1 = Feeding period 1, first collection; 5 P2 = Feeding period 1, second collection; 6 Pre2 = Pre-inoculation sampling 2; 7 Post2 = Post-inoculation sampling 2; 8 P3 = Feeding period 2, first collection; 9 P4 = Feeding period 2, second collection.

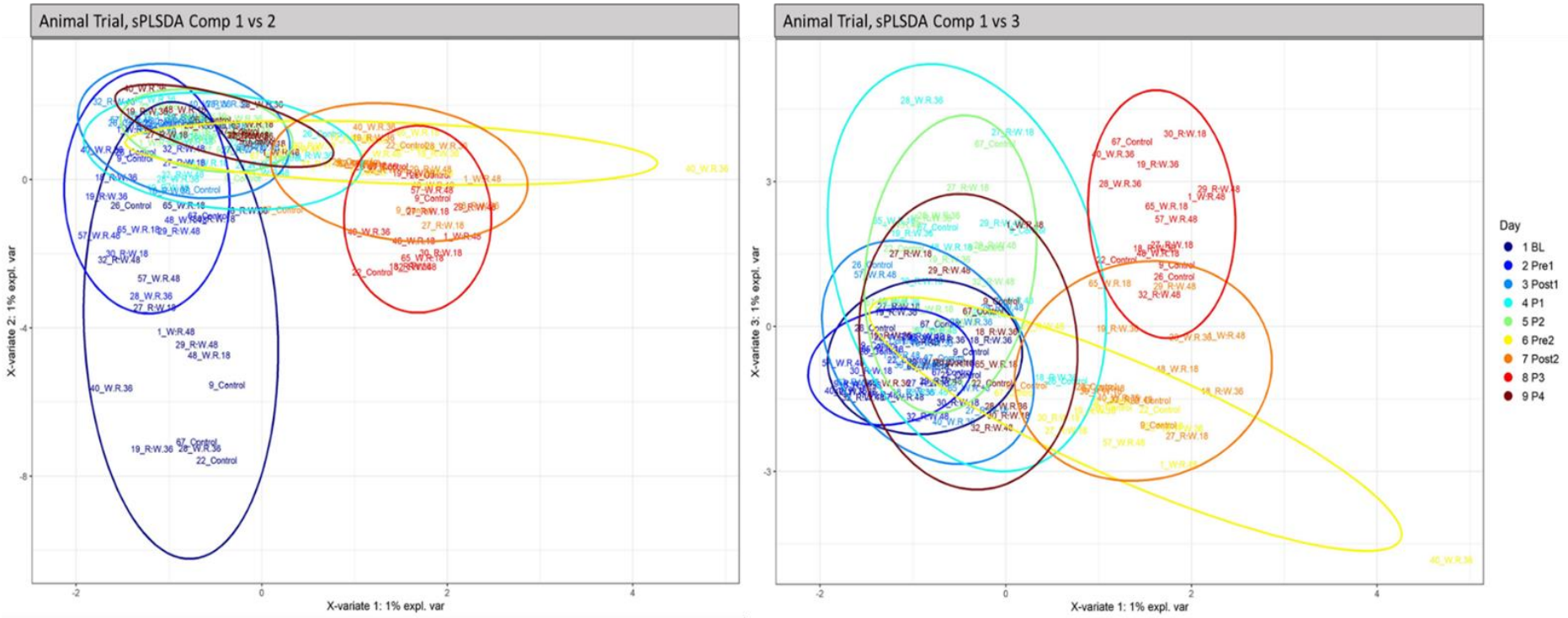


Figure 4.23. Clustered image map of all microbial families identified as contributing to differences occurring between sampling periods. Differences were determined from the Partial Least Squares (sparse) matrix of microbial families also used to generate the SPLSDA. The matrix is graphically represented with each entry of the matrix coloured on the basis of contribution (see colour key). The rows and columns are ordered according to a hierarchical clustering. Dendrograms resulting from the clustering are on left and to the top of the image, with a bar indicating the day. The nine sample collection sessions are coloured accordingly (1 BL= Baseline; 2 Pre1= Pre-inoculation sampling 1; 3 Post2= 3h post-inoculation sampling 1; 4 P1 = Feeding period 1, first collection; 5 P2 = Feeding period 1, second collection; 6 Pre2 = Pre-inoculation sampling 2; 7 Post2 = Post-inoculation sampling 2; 8 P3 = Feeding period 2, first collection; 9 P4 = Feeding period 2, second collection.

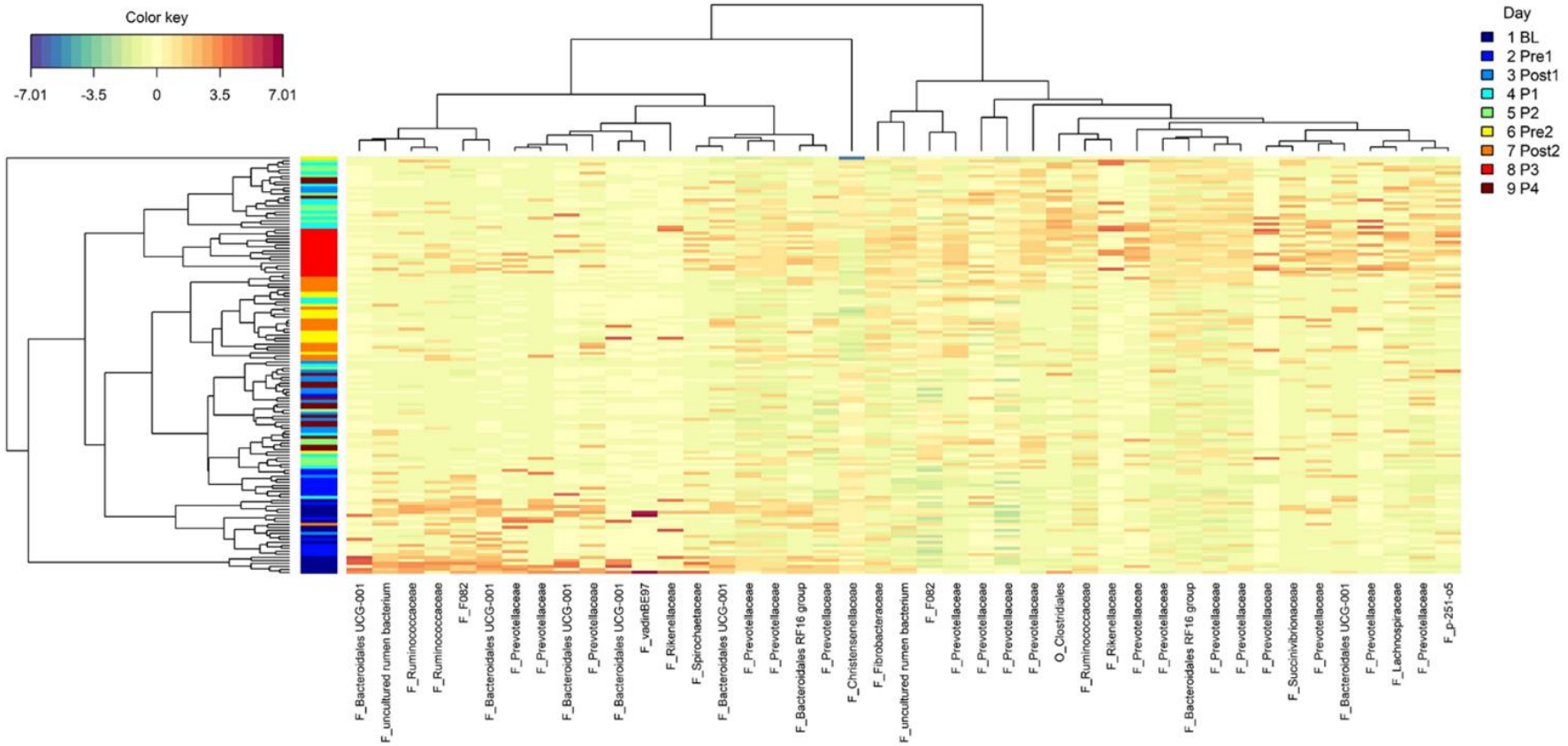


Figure 4.24. Partial Least Squares Discriminant Analysis (sparse) (sPLSDA, Components 1 vs 2 and Components 1 vs 3) of bacterial and archaeal populations of rumen samples from the animal trial, with the rumen samples collected pre- and post-inoculation excluded. Labelling of samples is on the basis of animal number_Diet1.Diet2.%Leucaena. Samples from the nine sample collection sessions are coloured accordingly (BL= Baseline; P1 = Feeding period 1, first collection; P2 = Feeding period 1, second collection; P3 = Feeding period 2, first collection; P4 = Feeding period 2, second collection.

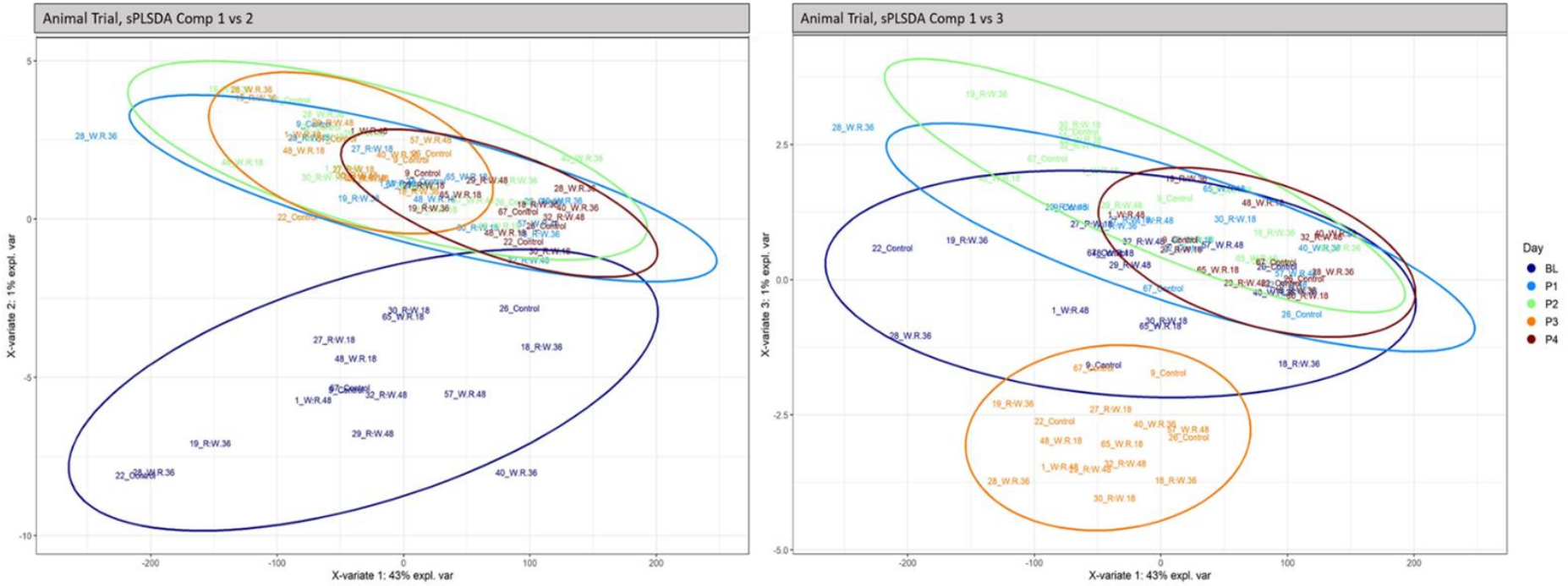
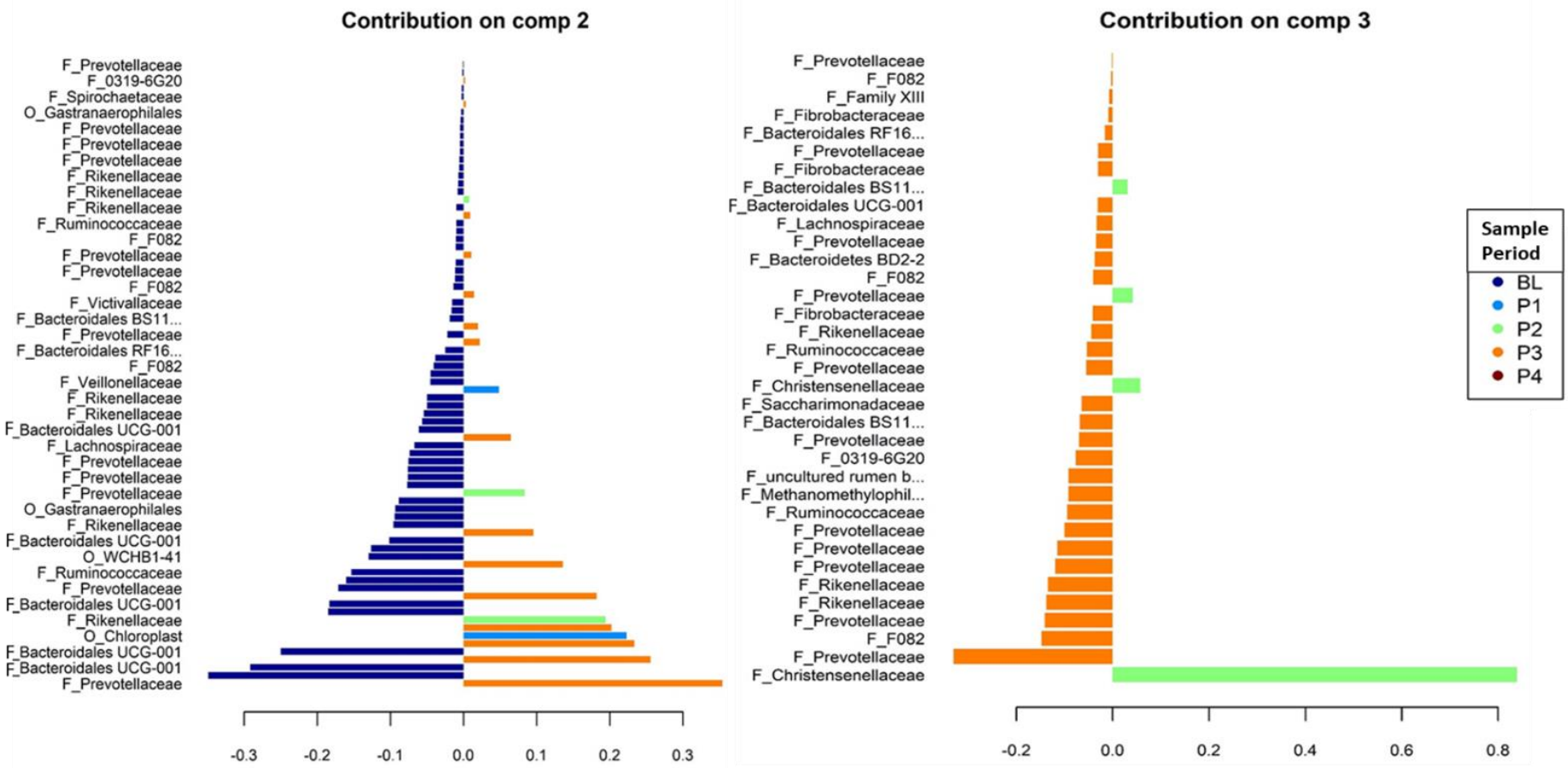


Figure 4.25. Contribution plots generated from the Partial Least Squares Discriminant Analysis (sparse) components 2 and 3, showing which microbial families contributed to the differences occuring between sample collection periods of the animal trial. Families are represented on bar plots, with the negative or positive correlation indicated by the direction of the bars in relation to the scale below each plot. Bars are also coloured according to the period to which they contributed the most effect. Longer family names are truncated and where the family name was not designated, the order is given.



4.1.2.3 Fermenter and Animal Trial Synergistes populations

A subset of sequences representing fermenter bacterial populations classified within the phylum Synergistetes was created from the bacterial and archaeal sequence dataset generated using the V4 16S rRNA gene primers. Similarly, a subset of phylum Synergistetes from the corresponding dataset from the animal trail was created. These subsets were used to determine if any changes occurred in the Synergistetes populations throughout the duration of the 30-day fermentations (Fig. 4.26), and throughout the animal trial (Fig. 4.27). This approach also gave an indication of how well the microbiome sequence dataset captured the presence and relative abundance of these bacterial communities. While the fermenter sequence dataset was able to detect and classify Synergistetes populations within every fermenter fluid sample, the animal trial sequence dataset only detected Synergistetes populations in 17 of the 139 rumen fluid samples included in the analysis. This indicated that either the phylum Synergistetes was at only very low concentrations within the rumen of the trial animals, or the animal trial sequence dataset was of insufficient sequence depth to enable detection of this phylum. The latter is not unlikely as microbial populations classified as the phylum Synergistetes are usually relatively minor, low abundance microbial populations within the rumen.

The sequence dataset generated for the Leucaena fermentations indicated that of the microbial populations classified within the overall phylum Synergistetes, populations of the genus Pyramidobacter were the most highly abundant. At the later days of all fermentations, populations of *Cloacibacillus* and *Synergistes*, including *S. jonesii*, were also detected. When the microbiome sequence results were compared to the qPCR assays specifically designed for the detection and enumeration of *S. jonesii* populations, there were differences in the results obtained. The qPCR assays appeared to be more sensitive, detecting *S. jonesii* populations in samples which the sequence analysis determined to be negative for the presence of *S. jonesii*.

Despite the discrepancy in the results obtained for the two methods, sequences representing the phylum Synergistetes from fermenter fluid samples and animal trial rumen samples were compared. A sequence alignment of all the representative sequences indicated that the dominant *Pyramidobacter* populations from the fermenter, found in 87 of the 88 fermenter fluid samples examined, were not the same as those detected in the animal trial rumen samples, with several point mutations occurring in the 16S rRNA gene sequences (Fig. 4.28). Similarly, the majority of the *Cloacibacillus* and *Synergistes* sequences detected in either the fermenter or animal trial rumen fluid samples also showed sequence variation. The exception was a single *S. jonesii* population, which was detected in one animal at one rumen fluid collection time-point. The representative 16S rRNA gene sequence for this population showed 100% nucleotide (nt) homology to the *S. jonesii* population detected in 22 of the fermenter fluid samples. The genus *Fretibacterium*, which was detected in a single animal trial sample, was not detected in any of the fermenter fluid samples.

Further sequence analysis of the Synergistetes populations involved a phylogenetic comparison based on the 16S rRNA gene V4 region. This analysis included reference sequences from known bacterial isolates classified within the phylum Synergistetes. This showed that the Synergistetes populations of the fermenter and animal trial were most like those previously isolated from gut environments, including the rumen. Interestingly, two reference 16S rRNA gene sequences from bacteria isolated from previous Leucaena fermentations (*Pyramidobacter* strain YE332 and *S. jonesii* strain YE330), were included in the phylogenetic analysis and were found to be highly related (> 99% homology) to *Pyramidobacter* and *S. jonesii* representative sequences obtained from fermenter fluid samples (Fig. 4.29).

This comparison also indicated that although the most abundant Synergistetes populations found in the Leucaena fermentations (*Pyramidobacter* and *S. jonesii*) were closely related phylogenetically, they were not exactly the same as those detected in the rumen of cattle during the animal trial. As the animal trial dataset may have underestimated the overall abundance of Synergistetes

populations, further work would be required to determine whether the microbial populations of the Leucaena inoculums could actually establish and proliferate in the rumen, and consequently enhance the breakdown of the Leucaena cultivar plant material and the toxins associated with the feeding Leucaena.

Figure 4.26. Changes in populations of the phylum Synergistetes for all replicate Leucaena cultivar fermentations, with samples ordered on the x-axis according to the fermentation cultivar supplied to each fermentation and the fermentation day. Results of the *S. jonesii* qPCR are also indicated (High = *S. jonesii* concentration > 10^6 cells/mL; Medium = *S. jonesii* concentration < 10^6 and > 10^5 cells/mL; Low = *S. jonesii* concentration < 10^5 cells/mL).

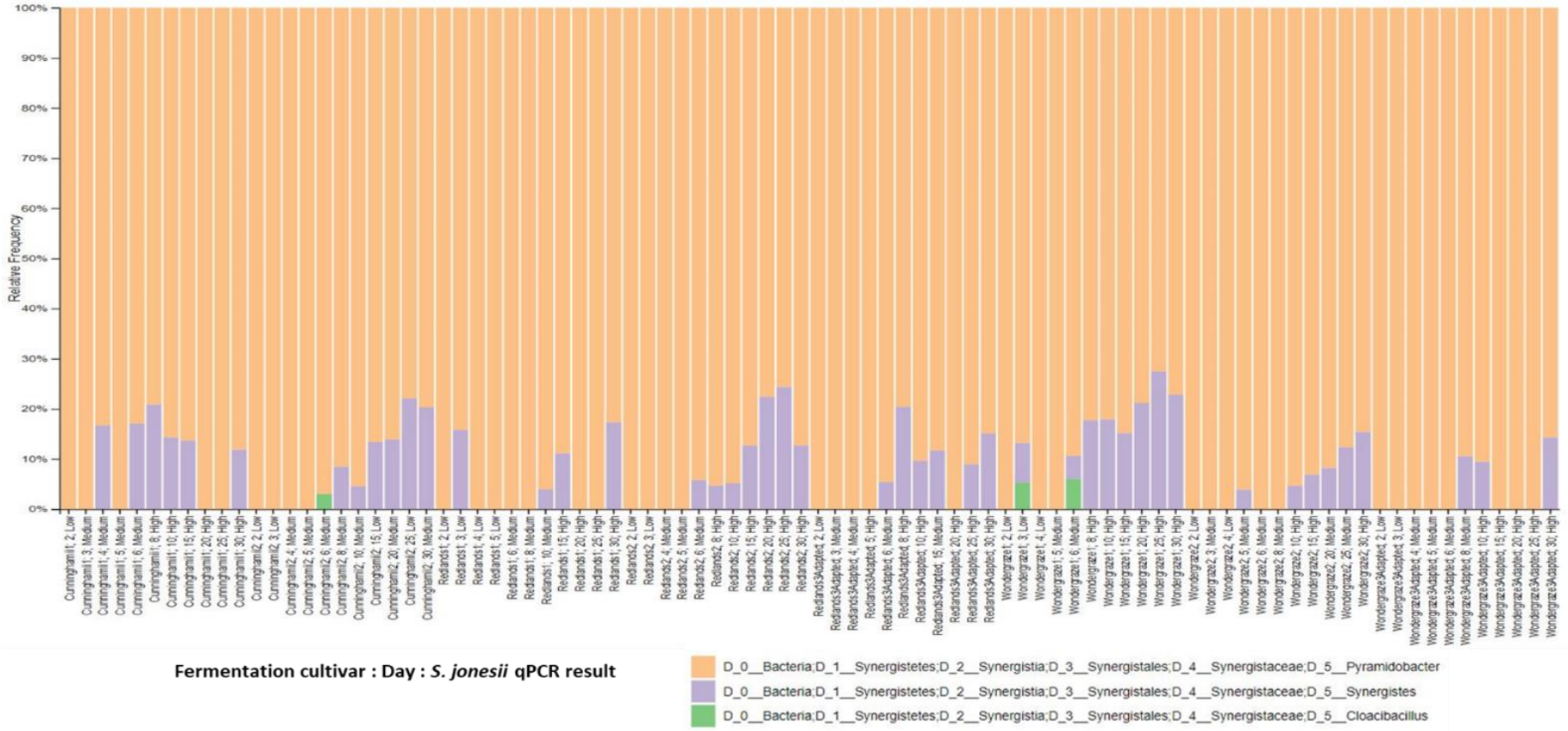


Figure 4.27. Populations of the phylum Synergistetes detected in all available animal trial samples. Rumen samples are ordered on the x-axis according to the animal trial sample collection period, the fermentation cultivar supplied in the diet, the percentage of the respective Leucaena cultivars added to the diet and the animal from which the rumen sample was collected.

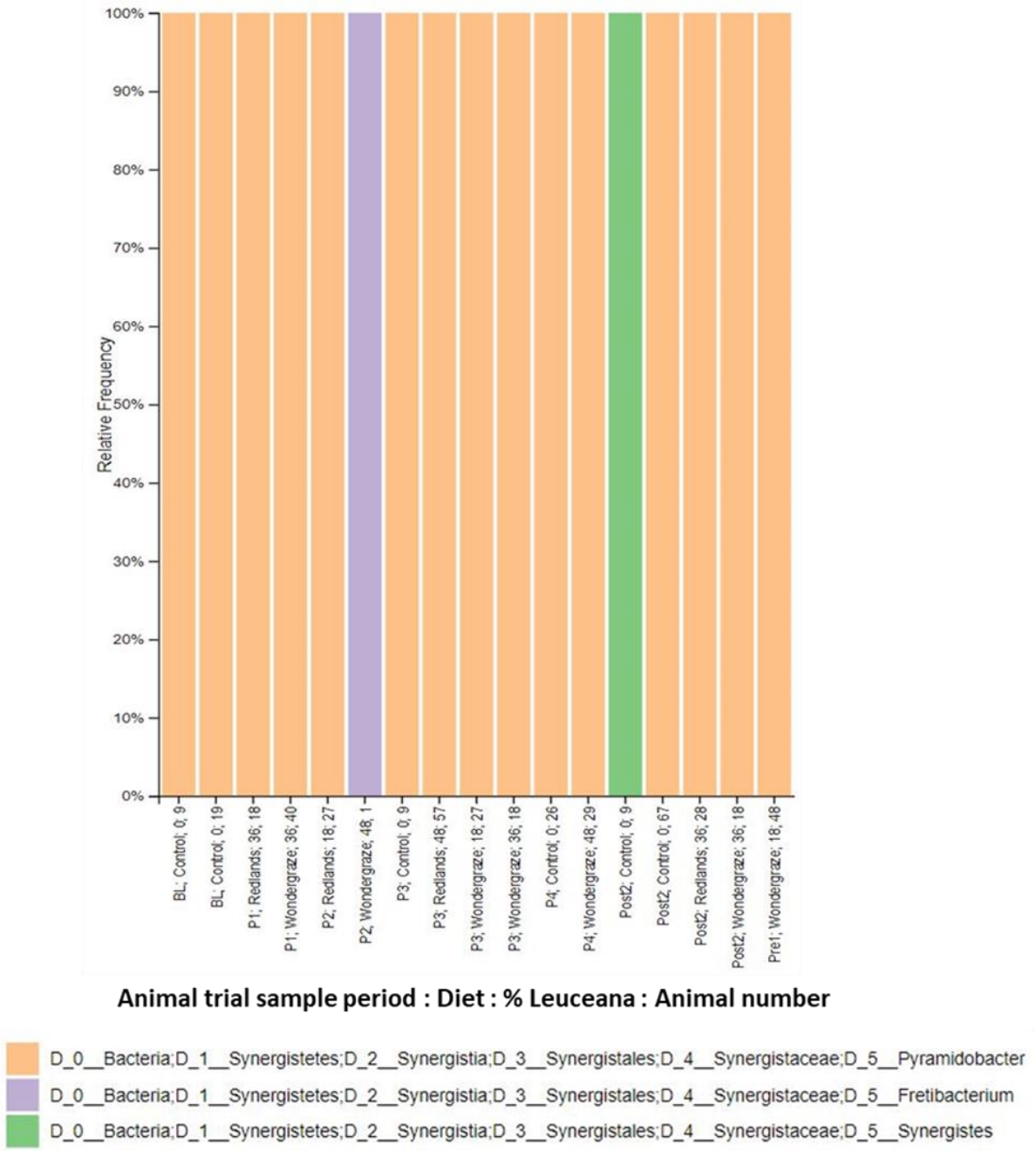


Figure 4.28. Alignment of 16S rRNA gene representative sequences taxonomically assigned as belonging to the phylum Synergistetes using the SILVA database (version 132). The 15 sequences are labelled and coloured according to whether they came from either the fermenter (F, gold), or animal trial (AT, green) sequence datasets; the sequencing variant they represent (a truncation of the name assigned using QIIME 2); genus and species level taxonomy assigned using the SILVA database; and the number of samples in which this sequence variant was observed (number shown in square brackets). The nucleotide alignment was generated using ClustalW (version 2.1) within Geneious (version 11.1.2) and differences in nt sequence are highlighted and a consensus sequence indicating the percentage of identity is included above the nt sequence alignment (● 100%; ● >30%; ● <30% identity).

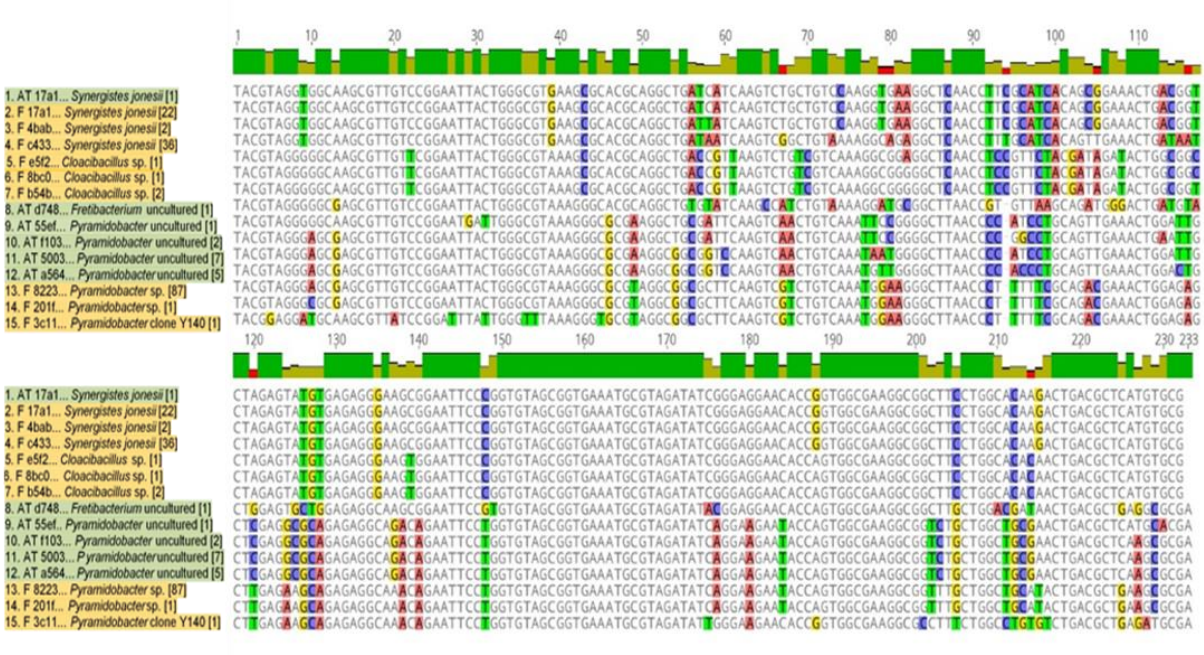
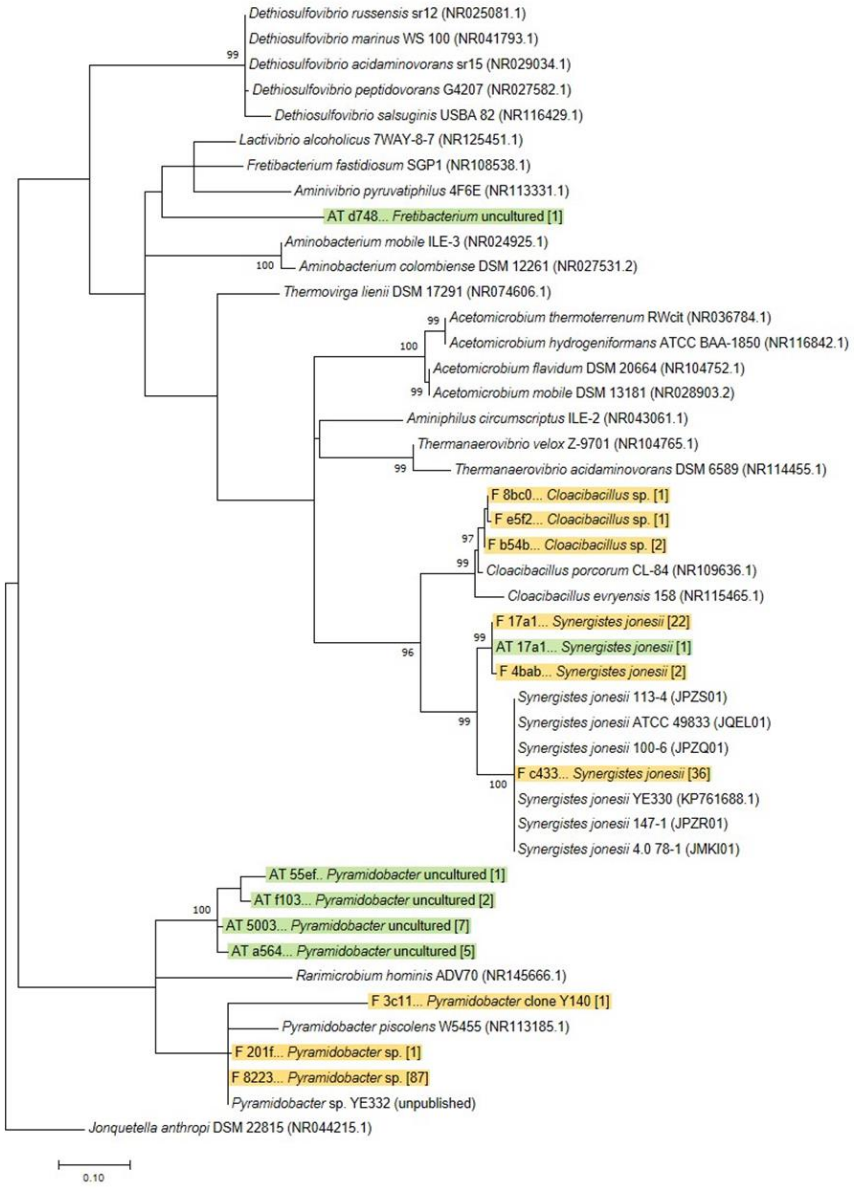


Figure 4.29. Maximum likelihood phylogeny of the 16S rRNA gene representative sequences from fermenter and animal trial samples found to belong to the phylum Synergistetes (45 sequences, 236 nucleotide positions included). The tree with the highest log likelihood (-2230.01) based on the Kimura 2-parameter model and a gamma distribution with 5 categories is shown. A rate variation model allowed for some sites to be evolutionarily invariable ([+I], 34.70% sites). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches, percentages <80% are not shown. The 16S rRNA gene sequence of Synergistetes species *Jonquetella anthropi* was used to root the tree. Representative sequences are coloured and labelled according to whether they came from either the fermenter (F, gold), or animal trial (AT, green) sequence datasets; the sequencing variant they represent (a truncation of the name assigned using QIIME 2); genus and species level taxonomy assigned using the SILVA 132 database; and the number of samples in which this sequence variant was observed (number shown in square brackets). The additional 16S rRNA sequences represent the major genera classified within the phylum Synergistetes, NCBI accession numbers are provided in parentheses. An unpublished 16S rRNA gene sequence for *Pyramidobacter* sp. strain YE332, previously isolated from a DAF Leucaena fermentation, is also included.



4.2 On-property survey for the prevalence of rumen detoxification of Leucaena toxins

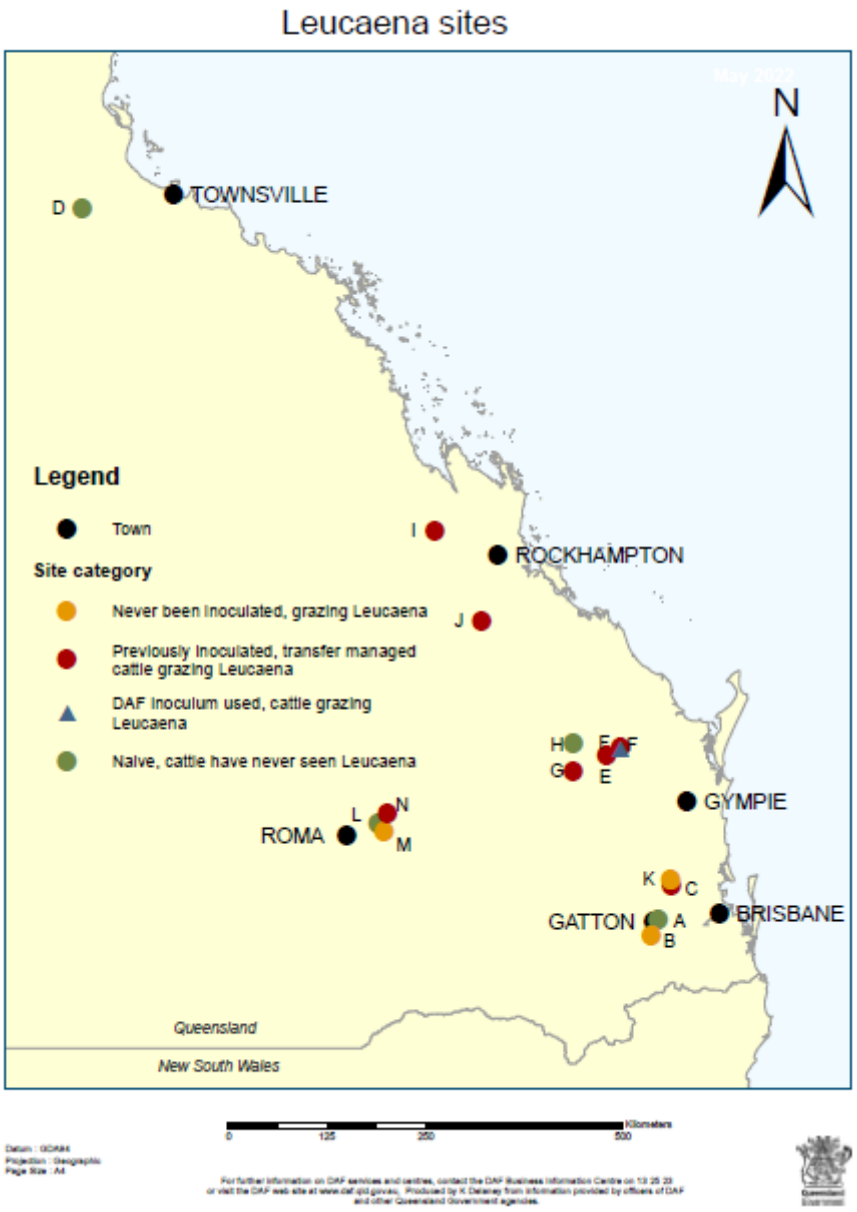
Following discussions with MLA in December 2021 the final six months of the project were re-scoped to include research with the aim to determine if populations of rumen bacteria able to completely degrade the toxic compounds in Leucaena are already present in Australian cattle. To address this, an on-property survey to determine the prevalence of naturally occurring detoxification of Leucaena toxins in the rumen of cattle was developed, with four treatments consisting of different production scenarios and the experimental unit being the property from which cattle were surveyed. The four treatments were: 1 – Properties where cattle have never been inoculated with the DAF inoculum but are grazed on Leucaena; 2 – Properties where cattle either received rumen fluid from the original CSIRO cattle or the fistulated cattle held at Brian Pastures Research Station (pre-1993) and have not been inoculated since with the DAF inoculum and are grazed on Leucaena; 3 – Properties where cattle have been inoculated with the DAF inoculum and are grazing Leucaena, and 4 – Properties that do not have Leucaena and their cattle have never been exposed to Leucaena (Naïve). The primary variable was the concentrations of the Leucaena-associated toxins, mimosine, 2,3-DHP and 3,4-DHP.

The DAF Animal Ethics Committee assessed and approved the application at their August 2021 meeting and the approval notification, SA 2021/08/796, is contained in Appendix 12.5. The Leucaena Network email to members and Future Beef bulletin article resulted in 24 producers registering their interest in participating in the on-property survey with five properties from South-East Qld (SEQ); 14 from Central Qld (CQ) and five from North Queensland (NQ). There were no responses received from producers whose cattle fitted into Treatment 2. A total of 14 properties (Sites) were visited, three in south-west Qld, four in south-east Qld, six in central Qld and one in north Qld with rumen samples collected from a total of 72 animals (Fig. 4.30). Details of the sites are summarised in Table 4.7.

Table 4.7. Summary of on-property survey sites visited. Treatment groups: 1 (Never inoculated, grazing Leucaena); 3 (DAF inoculum used or used previously and animals managed, grazing Leucaena) and 4 (Naïve cattle; never grazing Leucaena).

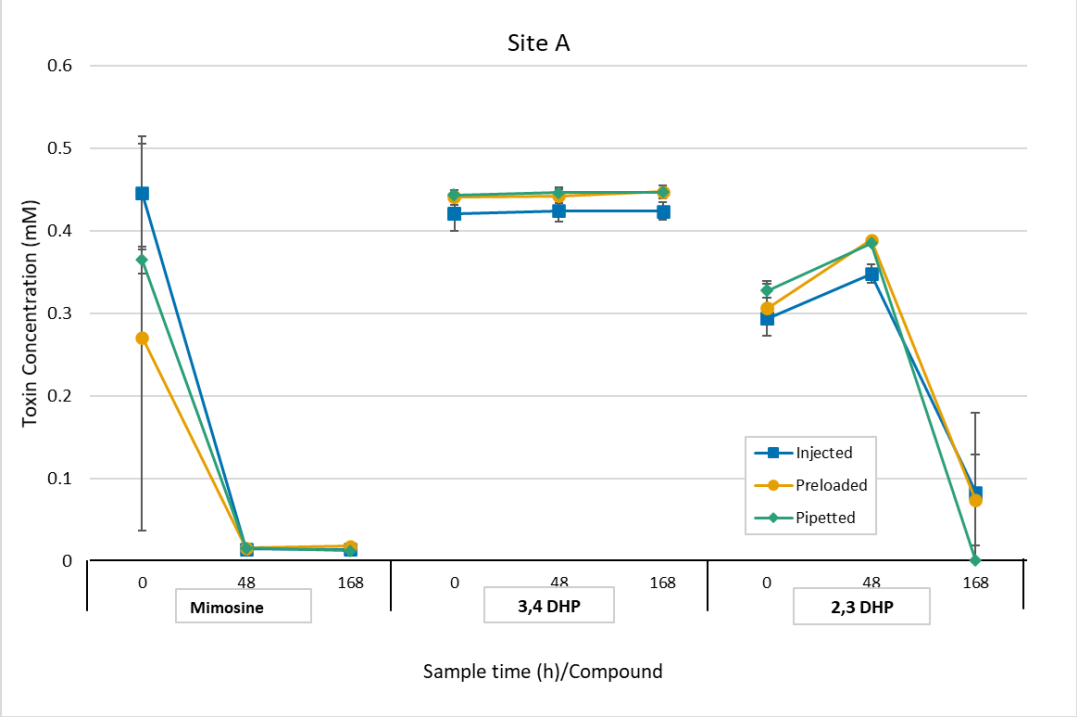
Site ID	Treatment Group	Location	Cattle class (No. sampled)	Leucaena variety	Pasture grass species
A	4	South-east Qld	Steers (4)	None	Kikuyu grass (*Pennisetum clandestinum*), Paspalum (*Paspalum notatum*), Ryegrass (*Lolium perenne*), cereal hay
B	1	South-east Qld	Cow (1), Heifer (1), Steer (2)	Old, unknown	Buffel Grass (*C. ciliaris*)
C	3	South-east Qld	Heifer (3), Steer (2)	Cunningham	Green Panic (*Megathyrsus maximum*)
D	4	North Qld	Steer (1)	None	Angleton bluegrass Floren (Dichanthium aristatum) Buffel Grass (*C. ciliaris*) Bambatsi panic (*Panicum coloratum sub. Makarikariense*)
E	3	Central Qld	Steer (4)	Cunningham	Green Panic (*M. maximum*), Bambatsi Panic (*P. coloratum sub. Makarikariense*), Rhodes grass (*Chloris gayana*)
F	3	Central Qld	Bull (6), Cow (3), Steer (1)	Wondergraze	Green Panic (*M.maximum*), Rhodes grass (*C. gayana*)
G	3	Central Qld	Heifer (1), Steer (1)	Wondergraze	Seca Stylo (*Stylosanthes scabra*)
H	4	Central Qld	Cow (4)	None	Buffel Grass (*C. ciliaris*)
I	3	Central Qld	Heifer (3), Steer (2)	Redlands, Wondergraze	Green Panic (*M. maximum*), Bambatsi Panic (*P. coloratum sub. Makarikariense*), Rhodes grass (*C. gayana*), Creeping Bluegrass (*Bothriochloa insculpta*), Angleton bluegrass Floren (*D. aristatum*)
J	3	Central Qld	Steer (4)	Cunningham	Green Panic (*M. maximum*), Buffel Grass (*C. ciliaris*) Rhodes grass (*C. gayana*)
K	1	South-east Qld	Cow (2), Heifer (2), Steer (1)	Cunningham	Spear grass (*Heteropogon contortus*), Rhodes grass (*C. gayana*)
L	4	South-west Qld	Heifer (5)	None	Green Panic (*M. maximum*), Buffel Grass (*C. ciliaris*), Bambatsi Panic (*P. coloratum sub. Makarikariense*) Creeping Bluegrass (*B. insculpta*)
M	1	South-west Qld	Cow (5)	Cunningham	Bambatsi Panic (*P. coloratum sub. Makarikariense*), Buffel Grass (*C. ciliaris*), Rhodes grass (*C. gayana*), Creeping Bluegrass (*B. insculpta*)
N	3	South-west Qld	Heifer (2), Steer (3)	Cunningham, Wondergraze	Green Panic (*M. maximum*), Buffel Grass (*C. ciliaris*), Bambatsi Panic (*P. coloratum sub. Makarikariense*) Creeping Bluegrass (*B. insculpta*)

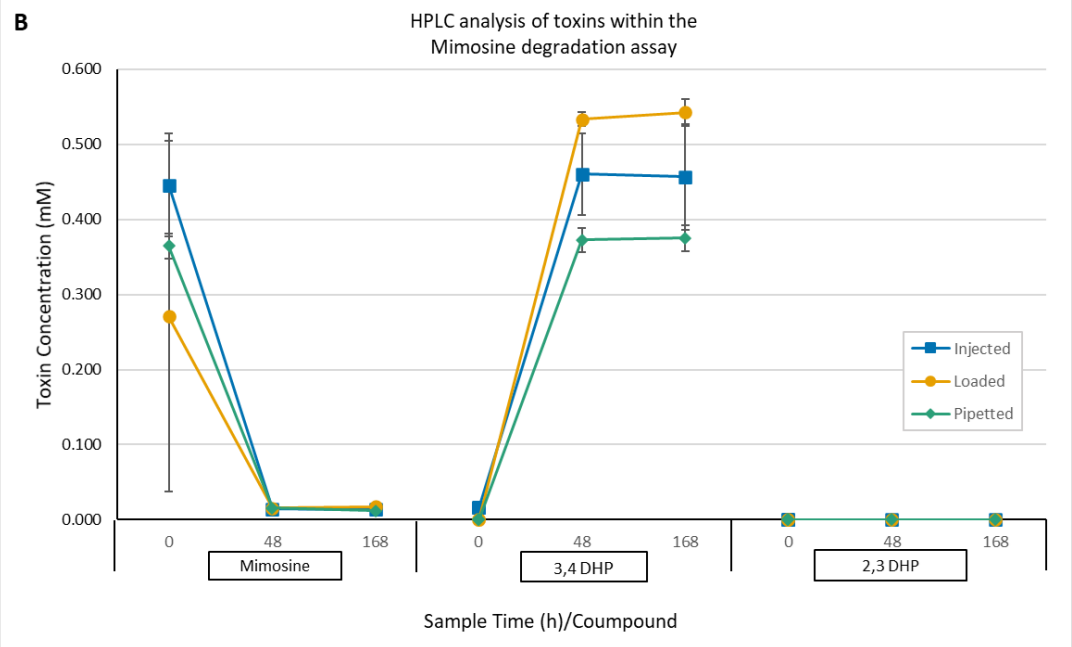
Figure 4.30. Map showing location of animals that have been sampled for the on-property survey to determine if cattle naturally possess Leucaena toxin-degrading rumen bacteria.



To test the most appropriate method for addition of the toxins to the rumen fluid samples, the performance of the 100 Ah lithium battery and portable incubators in the field, an initial rumen fluid sample was obtained from Site A which was classed as Treatment 4. This property does not have Leucaena and the cattle have never been exposed to Leucaena, as it was a rumen fistulated dairy steer located at the DAF Dairy Gatton. The three methods of addition of the toxins resulted in similar levels of toxin being delivered and the decision was made to use the injection method for future sample analysis. The rumen bacteria from the dairy steer were able to degrade the two toxins mimosine and 2,3 DHP but were unable to degrade the third toxin, 3,4 DHP (Fig. 4.31 A). To confirm this, the breakdown products produced within the mimosine degradation assay were analysed by HPLC, showing that as the mimosine was degraded, 3,4 DHP accumulated in the assay tubes, but was not broken down further into 2,3 DHP (Fig. 4.31 B).

Figure 4.31. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h within toxin degradation assays testing different methods of introducing the toxin (Injected, Preloaded, or Pipetted) to the rumen fluid sampled from the single Site A steer. A. Results of toxin degradation assays set up using different methods for addition of the toxins into the rumen fluid. B. Results of the three toxins within the mimosine degradation assay showing the degradation of mimosine resulting an increase in the toxic metabolite 3,4 DHP detected in the 48 h and 168 h samples.



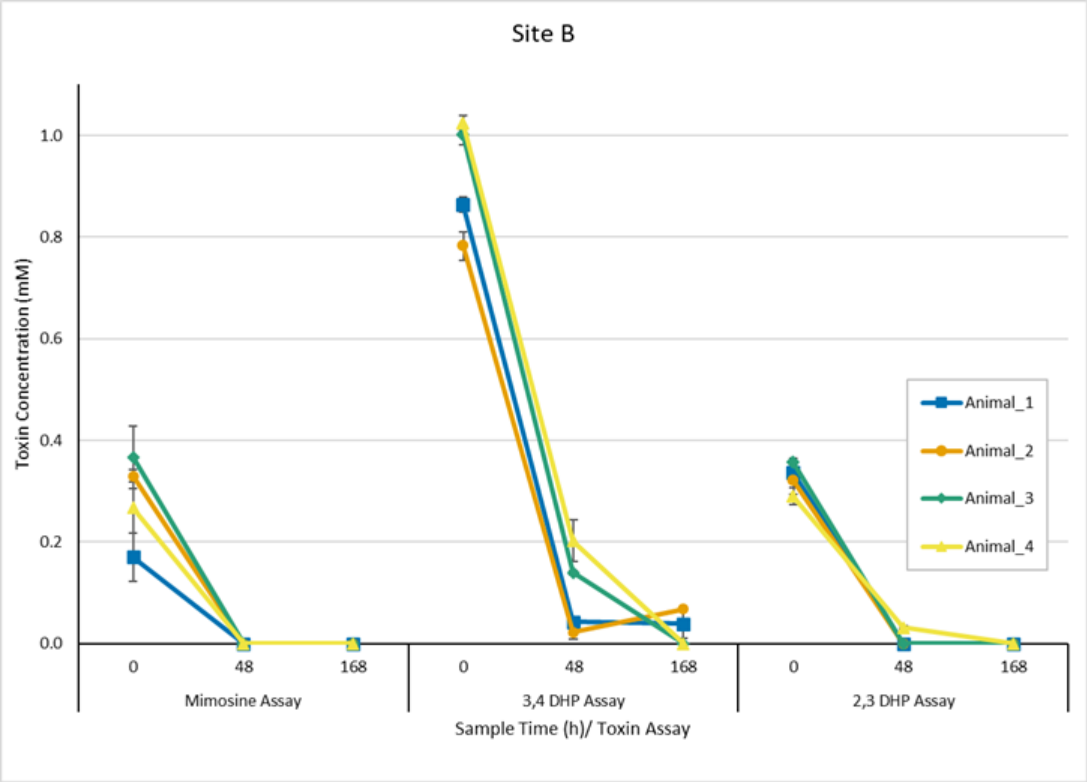


4.2.1 Treatment 1

Three properties, Sites B, K and M, were visited and cattle that had never been inoculated with the DAF Leucaena inoculum and grazing Leucaena, were rumen sampled.

The Site B property, located in south-east Qld, had an older stand of Leucaena (cultivar unknown) with Buffel Grass (*Cenchrus ciliaris*) pasture between the approx. 1 m rows. The producer was in the process of removing every second row of Leucaena to allow better pasture growth. Site B had a small herd of animals consisting of a mix of breed compositions with the majority of animals purchased and a few bred on property. The Leucaena stand was predominately used to fatten animals for feedlot entry. Four animals, one cow, one heifer and two steers, were rumen sampled and in the toxin degradation assays undertaken, all four were able to detoxify two of the toxins, mimosine and 2,3 DHP, within 48 h of incubation. In the 3,4 DHP toxin degradation assay two animals degraded all of the 3,4 DHP within 48 h and the remaining three animals completely degraded the 3,4 DHP within 168 h of incubation (Fig. 4.32).

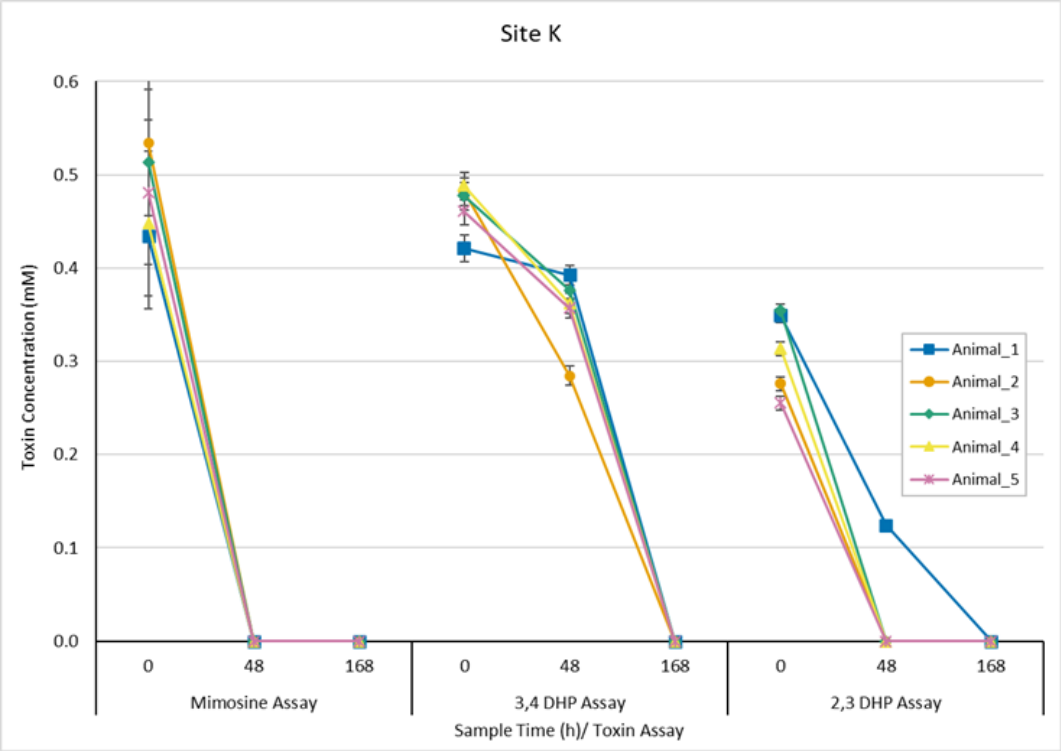
Figure 4.32. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h for toxin degradation assays set up using rumen fluid from four animals, two cows and two steers, sampled at the Site B.



The Site K property, located in south-east Qld, had a block of the Leucaena cultivar Cunningham planted at six to eight metre rows with mixed native Spear grass (*Heteropogon contortus*) and Rhodes grass (*Chloris gayana*) pasture. Cattle had free access to the Leucaena from pasture paddocks and whilst animals were occasionally kept out of the Leucaena block for six to eight weeks to allow the Leucaena to recover and achieve good regrowth, animals were never blocked in with the Leucaena. Five animals, two cows (Brahman cross, Charolais cross), two heifers (Charolais cross, Droughtmaster cross) and one steer (Charolais cross), were rumen sampled. The degradation assay showed they possessed rumen bacteria which were capable of completely degrading mimosine within 48 h. The rumen bacteria were only partially degrading 3,4 DHP after 48 h of incubation but it was completely degraded after one week of incubation. Four of the five animals were able to

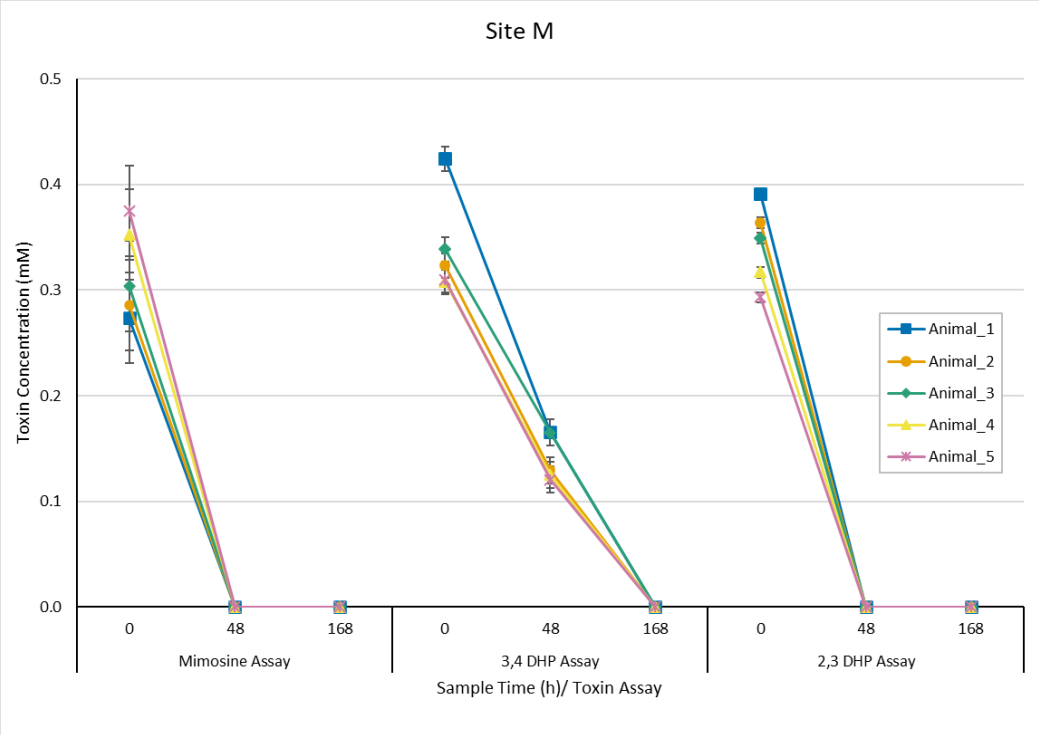
completely degrade 2,3 DHP within 48 h whilst one animal only completely degraded the 2,3 DHP after 168 h of incubation (Fig. 4.33).

Figure 4.33. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h for toxin degradation assays set up using rumen fluid from five animals, two cows, two heifers and one steer, sampled at Site K.



The property Site M, located in south-west Qld, had blocks of the Leucaena cultivar Cunningham, planted at eight metre rows, with inter-row mixed pasture consisting of Bambatsi Panic (*Panicum coloratum sub. Makarikariense*), Buffel Grass (*Cenchrus ciliaris*), Rhodes grass (*Chloris gayana*) and Creeping Bluegrass (*Bothriochloa insculpta*). Animals had access to the Leucaena blocks at all times from pasture only paddocks. The producer had previously used the DAF Leucaena inoculum but de-stocked during the drought. The property has been restocked with purchased cattle which have not been inoculated. Five Angus cows were rumen sampled and, in the degradation assays, all five animals possessed rumen bacteria able to detoxify two of the toxins, mimosine and 2,3 DHP, within 48 h of incubation. In the 3,4 DHP toxin degradation assay the rumen bacteria did not completely degrade all the 3,4 DHP within 48 h but it was able to completely degrade it within 168 h of incubation (Fig. 4.34).

Figure 4.34. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h in toxin degradation assays set up using rumen fluid from five cows, rumen sampled at Site M.



4.2.2 Treatment 2

This treatment included properties where cattle either received rumen fluid from the original CSIRO cattle or the fistulated cattle held at Brian Pastures Research Station (pre-1993) and were not inoculated since with the DAF inoculum. No properties fitting into the specifications for this treatment were identified.

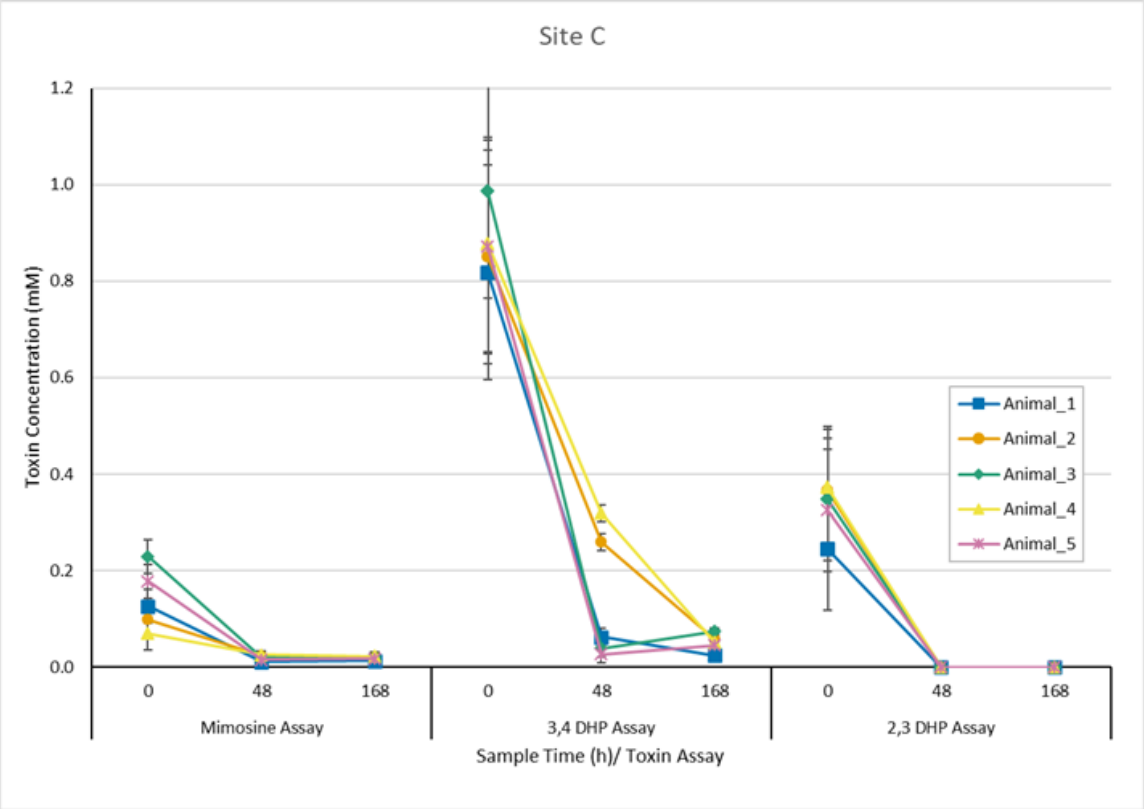
4.2.3 Treatment 3

Seven properties, Sites C, E, F, G, I, J and N, were visited where the cattle had either been inoculated with the DAF Leucaena inoculum or animals had been inoculated previously and the herd managed to maintain the presence of the detoxifying rumen bacteria. The animals sampled at these properties were grazing Leucaena.

The property Site C, located in south-east Qld, had blocks of the Leucaena cultivar Cunningham, planted at three metre rows, with the inter-row mixed pasture consisting of Green Panic (*Megathyrsus maximum*). The producers used a rotational grazing system and had inoculated animals previously and subsequently managed the passage of the toxin-degrading bacteria by mixing animals. The five animals sampled came from the Cunningham block where they had been on and off Leucaena for eight to ten months. Three Angus-Wagu F1 heifers and two Angus-Wagu steers were rumen sampled and, in the degradation assays, all five animals possessed rumen bacteria able to detoxify two of the toxins, mimosine and 2,3 DHP, within 48 h of incubation. The rumen bacteria were so efficient most of the mimosine (0.45 mM) added to the assay was degraded before the 0 h samples were able to be taken with a resulting peak of 3,4 DHP seen in the HPLC analysis of the mimosine time 0 h sample. In the 3,4 DHP toxin degradation assay three animals were able to completely degrade the 3,4 DHP within 48 h incubation. The rumen bacteria in two of the animals, a

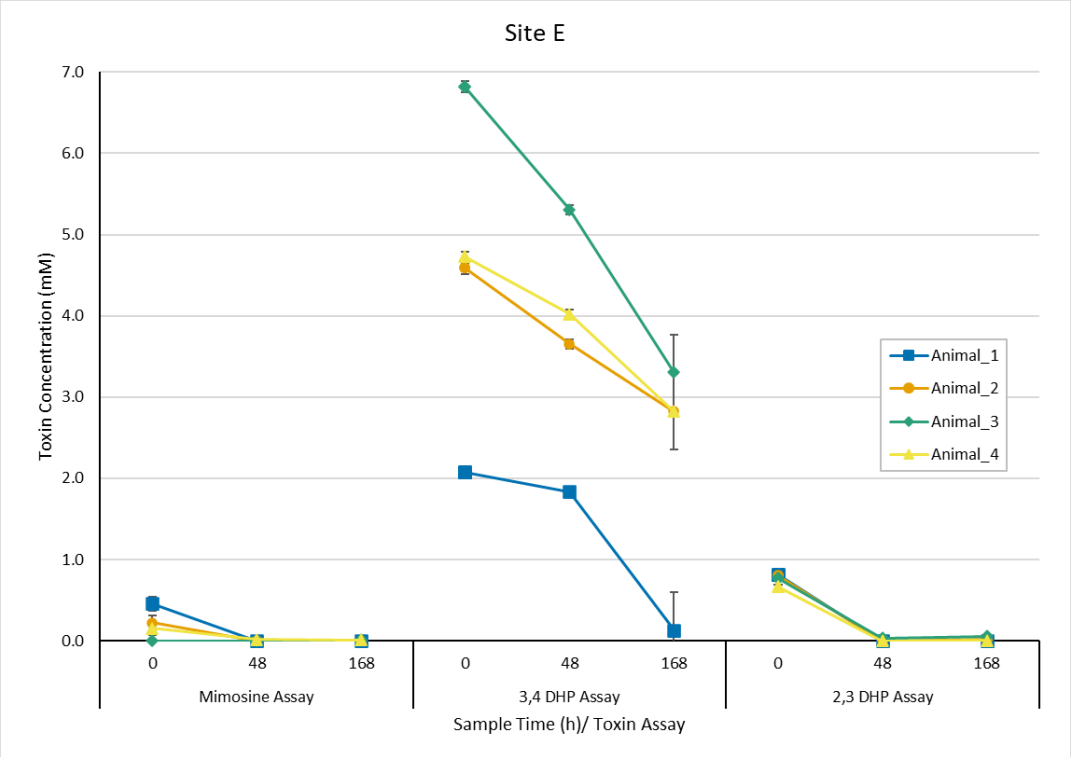
heifer and a steer, had degraded 65 % and 76 % of the 3,4 DHP respectively within 48 h and completely degraded it within 168 h of incubation (Fig. 4.35).

Figure 4.35. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h for the toxin degradation assays set up using rumen fluid from five animals, three heifers and two steers, rumen sampled at Site C.



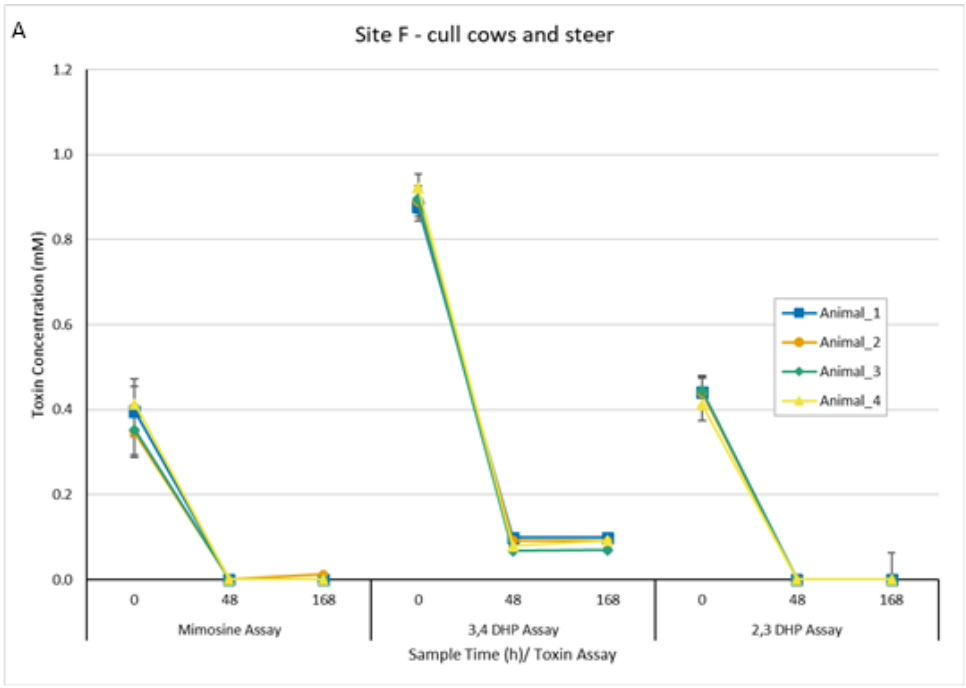
The property, Site E, located in central Qld, had blocks of the Leucaena cultivars Cunningham and Wondergraze, planted at six metre rows with the inter-row mixed pasture consisting predominantly of Green Panic (*Megathyrsus maximum*) with some Bambatsi Panic (*Panicum coloratum sub. Makarikariense*) and Rhodes grass (*Chloris gayana*). At Site E animals sampled included four Droughtmaster steers, which had been boxed in Leucaena Cunningham for approximately four to five weeks together with several cull cows from a herd that had previously been inoculated with the DAF rumen inoculum. In the rumen fluid samples collected from all four animals, visible Leucaena plant material was present. The degradation assays demonstrated that all animals possessed rumen bacteria capable of completely degrading the toxins mimosine and 2,3 DHP within 48 h of incubation. In the time 0 h samples taken from all the degradation assays, HPLC analysis revealed that all animals had very high levels of 3,4 DHP present, up to seven times higher than the amount of toxin added into the 3,4 DHP degradation assay. This indicated that whilst the animals were consuming Leucaena and degrading the mimosine they were not detoxifying the mimosine breakdown product 3,4 DHP, which was then accumulating in the rumen. Only one animal completely degraded 3,4 DHP after 168 h of incubation, the other three were showing incomplete degradation (Fig. 4.36). The lack of 3,4 DHP degradation indicates that the steers may not have been sufficiently interacting with the inoculated cull cows to enable between-animal transfer of the toxin-degrading rumen bacteria and could be at risk of poisoning. We recommended that the animals be drenched with the DAF inoculum to ensure they could effectively detoxify 3,4 DHP.

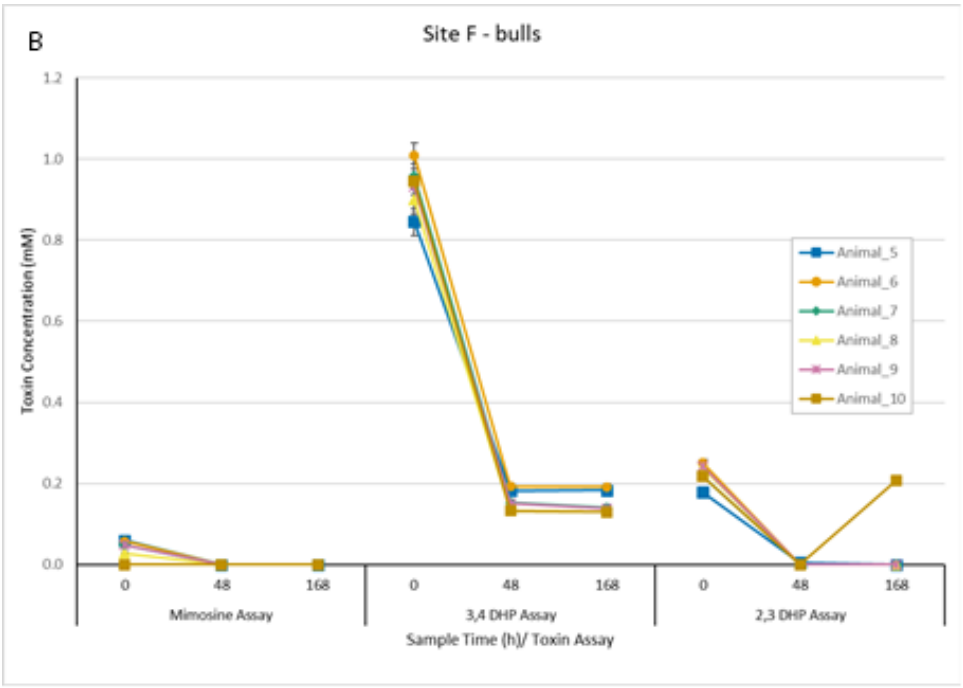
Figure 4.36. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h for the toxin degradation assays set up using rumen fluid from four steers, rumen sampled at Site E.



Property Site F, located in central Qld, had blocks of the Leucaena cultivar Wondergraze, planted at six metre rows, with inter-row pasture consisting of predominately Green Panic (*Megathyrsus maximum*) with some Rhodes grass (*Chloris gayana*). Two groups of animals were sampled at Site F, animals 1 to 4 were three cull cows and one steer that had been managed through transfer from co-grazing with previously inoculated animals. The second group of six animals were Droughtmaster bulls grazing Leucaena with 10 % of the bulls having received the DAF Leucaena inoculum approximately two weeks prior to our visit and were actively grazing Leucaena. The toxin degradation assays demonstrated that all 10 animals sampled, possessed rumen bacteria capable of completely degrading the toxins mimosine and 2,3 DHP within 48 h (Fig. 4.37). Animal 10 had measurable 2,3 DHP in the 168 h sample of the 2,3 DHP degradation assay which was unexpected as the 48 h sample indicated that it had been completely consumed. In the 3,4 DHP degradation assays all 10 animals degraded the majority of the 3,4 DHP (76 % to 86 %) and did not completely degrade the 3,4 DHP after 168 h of incubation.

Figure 4.37. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h for the toxin degradation assays set up using rumen fluid from A. three cows and one steer and B. six bulls, rumen sampled at Site F.



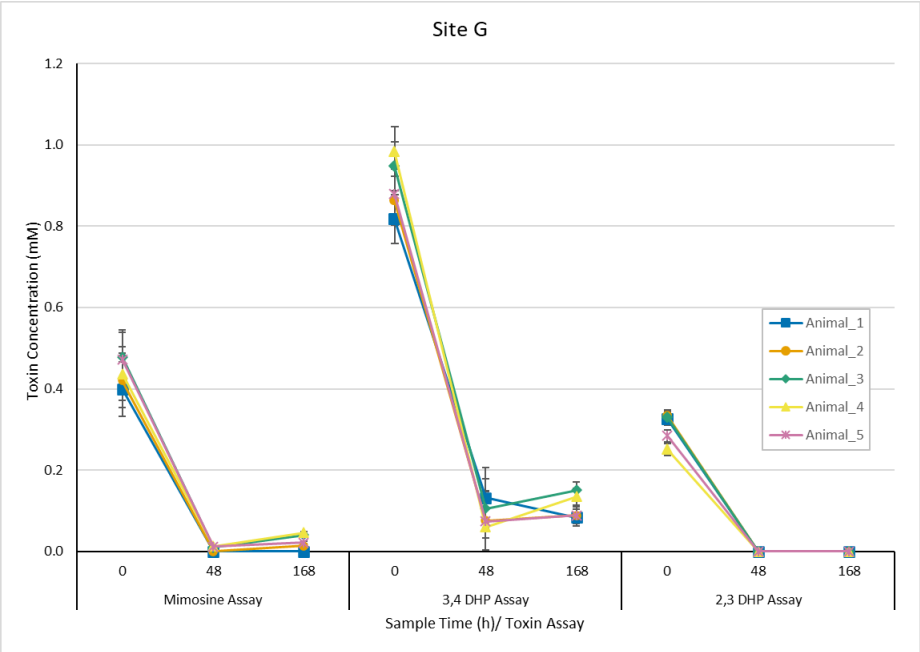


Property, Site G, located in central Qld, had blocks of the Leucaena cultivar Wondergraze, planted at 12 m row spacing, with inter-row pasture consisting of predominately Seca Stylo (*Stylosanthes scabra*). Cattle have access to Leucaena year-round (hedged) and supplemented with phosphorus lick blocks. The producer inoculated the herd with the DAF Leucaena inoculum approximately eight years ago and has managed the transfer between groups of incoming animals since. The five cattle sampled were five steers (two Santa crosses, a Murray grey cross and two Angus) and one pure Angus heifer. The toxin degradation assays demonstrated that all five cattle sampled possessed rumen bacteria capable of completely degrading the toxins mimosine and 2,3 DHP within 48 h. In

the 3,4 DHP degradation assays, all animals degraded the majority of the 3,4 DHP (84 % to 96 %) within 48 h. In some of the 3,4 DHP assays, three animals had increased levels of 3,4 DHP in the samples taken after 168 h of incubation (Fig. 4.38).

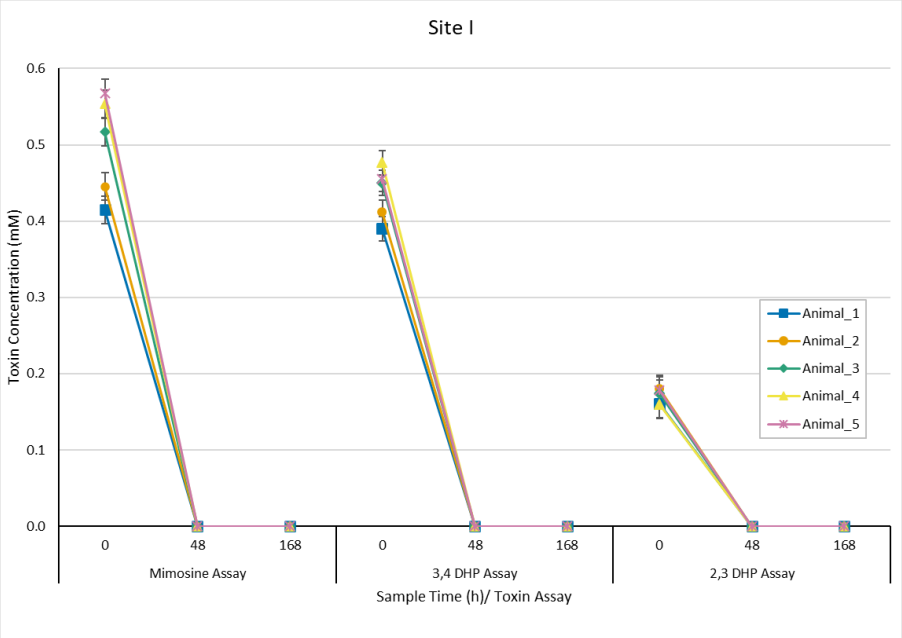
One explanation for this slight increase in 3,4 DHP may be related to potential technical errors, identified after assays were complete. There is not a commercial source of 3,4 DHP available, therefore commercially available mimosine is routinely used to synthesise 3,4 DHP 'in-house'. The toxin can be difficult to dissolve in water and occasionally there may be undissolved crystals, which may have inadvertently transferred into the degradation assay tubes, thus increasing the concentrations delivered. Another explanation may be related to technical problems being experienced with the HPLC equipment used to measure the concentration of the toxins in these site samples.

Figure 4.38. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h for the toxin degradation assays set up using rumen fluid from four steers and one heifer, rumen sampled at Site G.



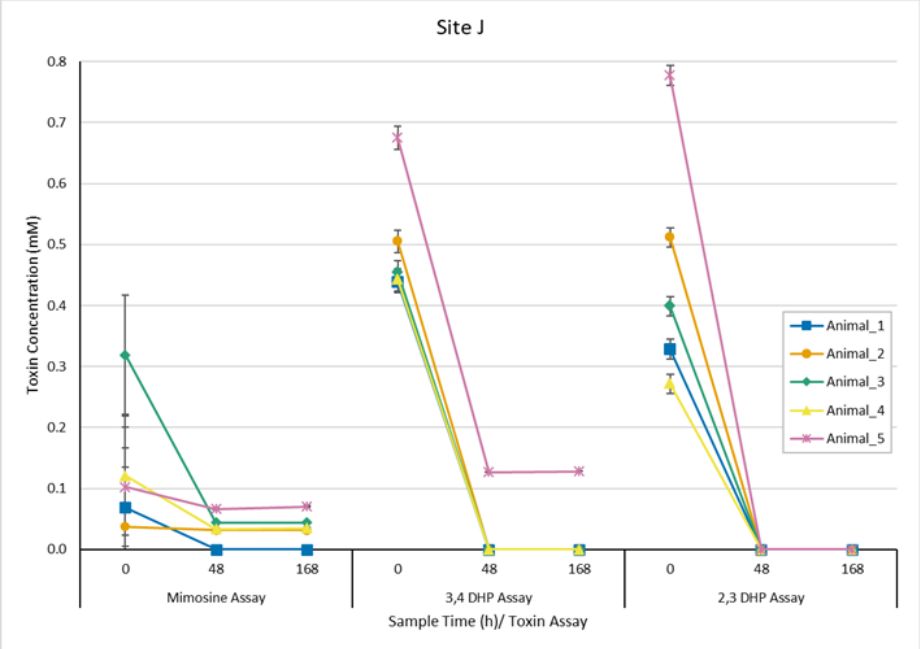
Property, Site I, located in central Qld, had blocks of the Leucaena cultivars Wondergraze and Redlands, planted at 12 m row spacing, with inter-row pasture consisting of Green Panic (*Megathyrsus maximum*), Bambatsi Panic (*Panicum coloratum sub. Makarikariense*), Rhodes grass (*Chloris gayana*), Creeping Bluegrass (*Bothriochloa insculpta*) and Floren (*Dichanthium aristatum*). The producer last inoculated animals with the DAF inoculum in August 2018 and has managed the transfer between groups of animals since. From the herd grazing the Leucaena, the producer retains a number of animals, usually cull heifers or cows and the tail of the feeder steers not at sale or feedlot entry weight, mixing these animals with the new feeder steers or weaner heifers. The five animals sampled consisted of three heifers (two Droughtmaster cross, one Charolais cross) and two Droughtmaster cross steers. The degradation studies demonstrated that all five animals possessed rumen bacteria capable of degrading the three toxins within 48 hours (Fig. 4.39). This indicates that the management practices used were successful at transferring the toxin-degrading rumen bacteria to new animals.

Figure 4.39. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h for the toxin degradation assays set up using rumen fluid from three heifers and two steers, rumen sampled at Site I.



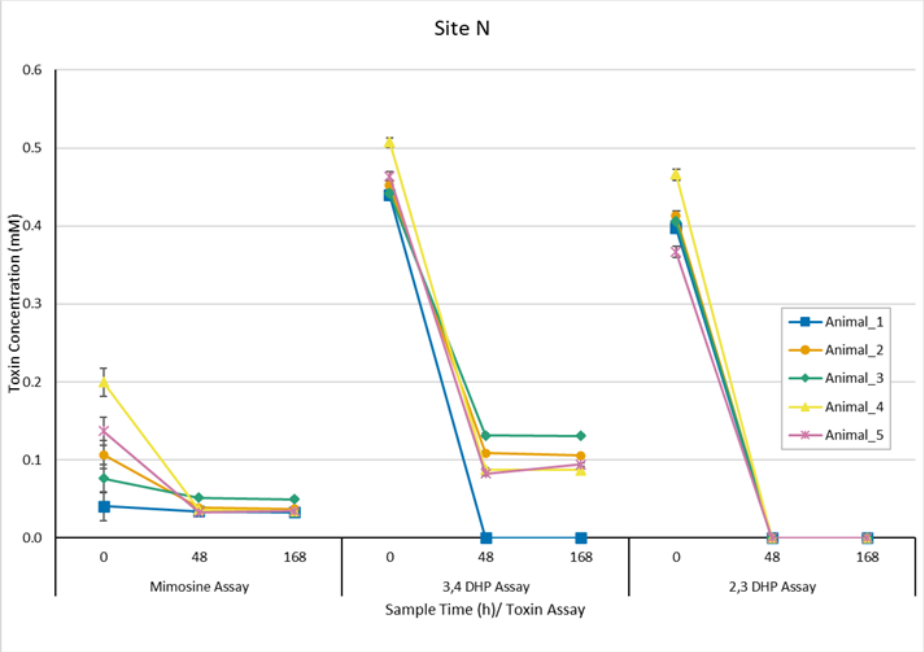
Property, Site J, located in central Qld, had blocks of the Leucaena cultivar Cunningham, planted at six metre double-row spacing, with inter-row pasture consisting of Green Panic (*Megathyrsus maximum*), Buffel Grass (*Cenchrus ciliaris*) and Rhodes grass (*Chloris gayana*). The producer runs feeder steers for weight gain prior to going into a feedlot. They used the DAF Leucaena inoculum in 2017 and managed the transfer by mixing new animals with current animals, as one mob, for several weeks prior to drafting out animals for transport to the feedlot. In 2020, the property received good rain resulting in the herd grazing pasture. During this period the cattle had no time grazing the Leucaena which was also not cut during this time. The producer was concerned that the Leucaena toxin-degrading rumen bacteria had been lost as the current mob of 12-month-old Wagu steers had not gained weight as expected in the previous months grazing the Leucaena. The height of the Leucaena may have restricted the ability of the steers to access leaf material. Five steers were sampled and in the degradation assays only one steer demonstrated it possessed rumen bacteria able to degrade all three toxins within 48 h of incubation (Fig. 4.40). Interestingly the other four animals' rumen bacteria did not degrade the mimosine completely within the assay but were able to degrade the 2,3 DHP within 48 h. Three of the steers were also able to degrade the 3,4 DHP within 48 h. Animal 5 had higher levels of mimosine and 3,4 DHP measured in the time 0 h samples of all three toxin degradation assays indicating that it was eating Leucaena but not effectively degrading the toxins. The lack of mimosine breakdown is an unusual result as there are many bacteria within the rumen that can degrade mimosine and it is usually the first toxic metabolite 3,4 DHP, that is unable to be degraded.

Figure 4.40. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h for the toxin degradation assays set up using rumen fluid from five steers, rumen sampled at Site J.



Property, Site N, located in western Qld, had blocks of the Leucaena cultivars Cunningham and Wondergraze, planted at either 12 m, eight to nine, or six metre row spacing, with inter-row pasture consisting of Green Panic (*Megathyrsus maximum*), Buffel Grass (*Cenchrus ciliaris*), Bambatsi Panic (*Panicum coloratum sub. Makarikariense*) and Creeping Bluegrass (*Bothriochloa insculpta*). The producer had, in 2012, borrowed cattle from a neighbouring property which had received the DAF inoculum and ran them with his cattle to transfer the toxin degrading rumen bacteria and managed by mixing carry-over cattle with new animals going into the Leucaena blocks. Three steers and two heifers, predominately Angus cross, grazing the Leucaena cultivar Wondergraze were rumen sampled. In the degradation assays all five animals possessed rumen bacteria The rumen bacteria efficiently degraded most of the mimosine (0.45 mM) added to the assay before the 0 h samples were taken with a resulting peak of 3,4 DHP seen in the HPLC analysis of the mimosine time 0 h sample. The rumen bacteria were able to completely degrade the mimosine and 2,3 DHP within 48 h of incubation but only one steer demonstrated it possessed rumen bacteria able to degrade all three toxins within 48 h of incubation (Fig. 4.41). The other four animals' rumen bacteria degraded the 3,4 DHP (70 to 83%) within 48 h but did not completely degrade it after 168 h of incubation.

Figure 4.41. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h for the toxin degradation assays set up using rumen fluid from three steers and two heifers, rumen sampled at Site N.

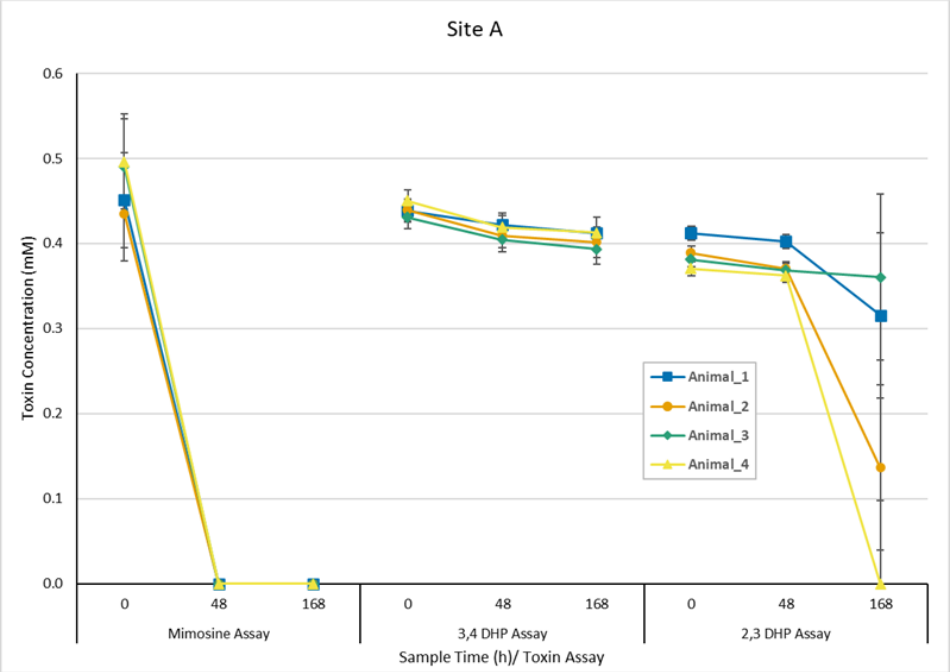


4.2.4 Treatment 4

Four properties, Sites A, D, H and L, were identified as meeting the specifications of Treatment 4. These properties did not have Leucaena planted and cattle which had never been inoculated or exposed to Leucaena (classed as Naïve cattle for the purposes of this study).

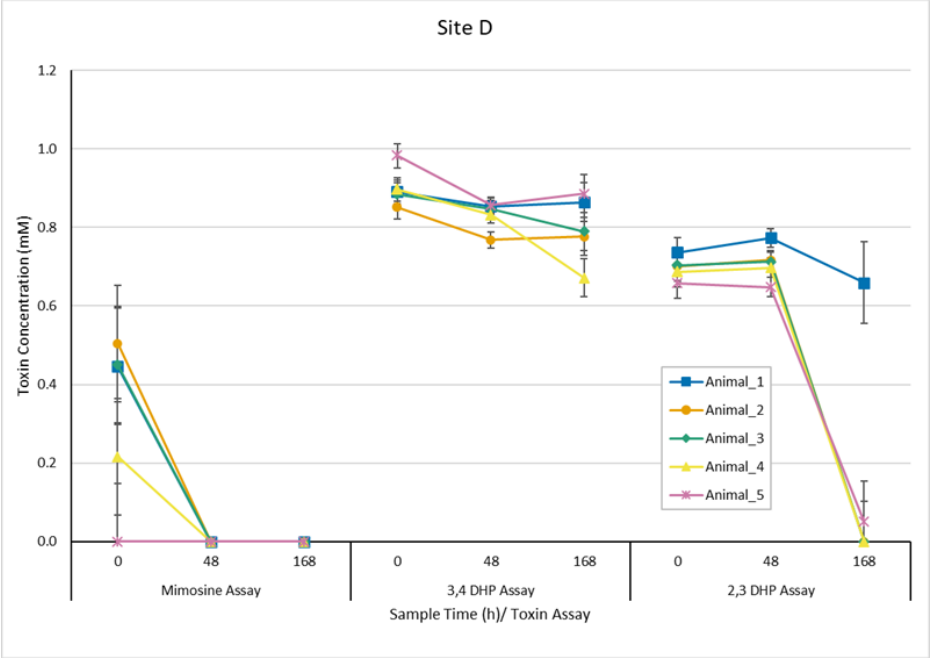
Property Site A, located in south-east Qld, had mixed pastures consisting of Kikuyu grass (*Pennisetum clandestinum*), Ryegrass (*Lolium perenne*) and the four Holstein Friesian steers rumen sampled were receiving supplementary cereal hay. All four steers possessed rumen bacteria that completely degraded the mimosine in 48 h. None of the animals were able to degrade 3,4 DHP after 168 h of incubation. In the 2,3 DHP degradation assay, the rumen bacteria had not degraded the 2,3 DHP after 48 h of incubation. However, after 16 h of incubation, the rumen bacteria of steer 4 had completely degraded the toxin 2,3 DHP, steers 1 and 2 partially degraded it and the rumen bacteria of steer 3 was unable to degrade 2,3 DHP (Fig. 4.42).

Figure 4.42. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h for the toxin degradation assays set up using rumen fluid from four steers, rumen sampled at Site A.



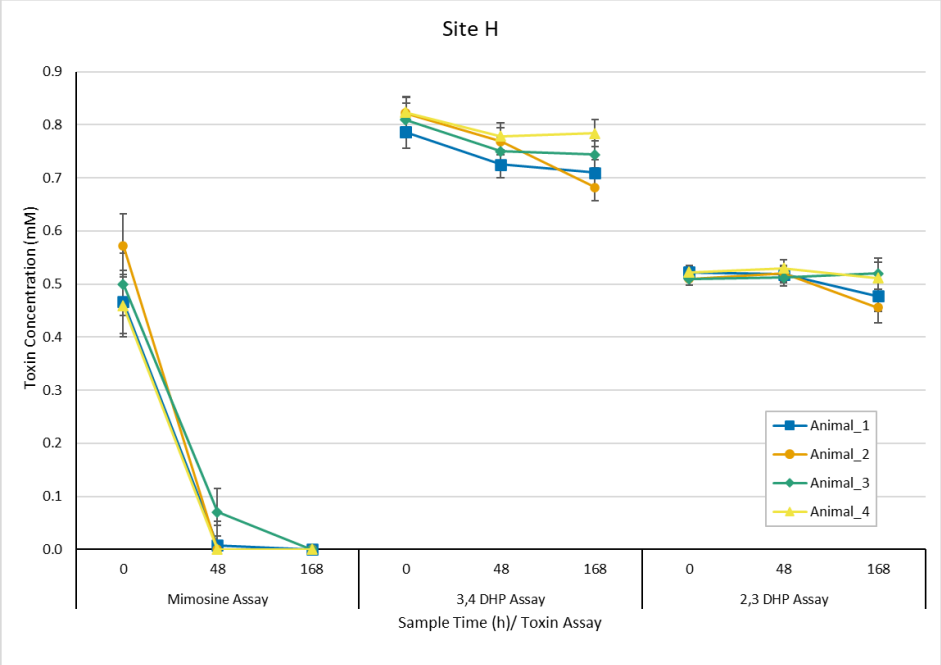
Property Site D, located in north Qld, had mixed native pastures and Droughtmaster composite cattle. Four steers were rumen sampled and all possessed rumen bacteria that completely degraded the mimosine in 48 h. None of the animals were able to degrade 3,4 DHP. In the 2,3 DHP degradation assay none was degraded after 48 h of incubation but after 168 h of incubation three of the four steers were able to degrade the 2,3 DHP toxin completely (Fig. 4.43).

Figure 4.43. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h for the toxin degradation assays set up using rumen fluid from five steers, rumen sampled at Site D.



Property Site H, located in central Qld, had a herd of Brangus cattle grazing Buffel Grass (*Cenchrus ciliaris*) pasture without supplementation. Four lactating cows were rumen sampled and all four possessed rumen bacteria that completely degraded the mimosine in 48 h but were not able to degrade either of the Leucaena toxic metabolites 3,4 DHP or 2,3 DHP (Fig. 4.44).

Figure 4.44. Concentrations of mimosine, 3,4 DHP and 2,3 DHP, as determined by HPLC, in samples taken at Times 0 h, 48 h and 168 h for the toxin degradation assays set up using rumen fluid from four cows, rumen sampled at Site H.



The on-property survey toxin degradation assay results for the 72 animals sampled across the 14 sites are summarised in Table 4.8.

4.2.5 Molecular analysis of on-property survey rumen samples

The microbial gDNA extracted from the 1.0 mL rumen samples collected from each animal were used to measure the *S. jonesii* populations, using quantitative PCR assay and all were below the assay's detectable limits. The nested PCR approach of Graham et al. (2013) was also undertaken, with PCR products only produced for *S. jonesii* 10^{10}cells/mL gDNA, *S. jonesii* 10^5 cells/mL gDNA positive controls and Fermentation 3 day 22 gDNA.

To increase the *S. jonesii* PCR assay sensitivity, the RNA present in rumen samples from either rumen fluid pellets (centrifuged, pelleted and frozen immediately) or RNA protected rumen fluid (centrifuged, pelleted, resuspended and stored frozen in RNA Protect solution), of selected on-property animals, and day 16 of Fermentation 17 fermentation fluid pellet or RNA protected fermentation fluid pellet were extracted (described in Section 3.2.2). The cDNA was then synthesised and used as template in the nested (two-step) PCR, following the methodology of McSweeney et al. (2019) with a positive control of *S. jonesii* gDNA extracted from 10^3 cells included. Results from the 2nd PCR assay showed that the *S. jonesii* positive control produced, as expected, a single band at approx. 800 bp (Fig. 4.45 lane 2 and 24).

The majority of the gDNA tested however, did not result in amplification products being generated by the nested PCR assays. The exceptions of were a strong band of the correct product size from the Fermentation 17 day 16 gDNA (Fig. 4.45, lane 12) and a very faint band corresponding to the Site B animal 2 gDNA sample (Fig. 4.45, lane 20).

When RNA from rumen fluid samples of Site F animals 6 and 7 and Site B animal 2, was used in the nested PCR assay, multiple, non-specific amplification products were generated, some of which appeared to be of the correct size (Fig. 4.45). Further testing of both the gDNA and cDNA in the *S. jonesii* quantitative PCR assay, showed that samples which were expected to amplify correctly as they were known to contain *S. jonesii* cells, i.e. the Fermentation 17 day 16 and *S. jonesii* 10^3 cells/mL gDNA positive control sample, did successfully amplify as expected. However, all other rumen fluid-derived samples tested, either amplified below the assay's level of detection of cycle 30 (10^3 cells/mL) or did not amplify at all. Therefore, this methodology was not used for the detection of *S. jonesii* in either rumen fluid or fermenter samples obtained in this investigation.

Further work will be required to better characterise the microbiome and *Synergistes* populations contained in both rumen fluid and fermenter samples. Alternative methodology, based on microbial genomics, using high through-put sequencing of the 16S rRNA gene (Illumina NextSeq platform) for characterisation of bacterial and archaeal populations, as well as shot-gun metagenomics (Illumina NovaSeq platform), to characterise the entire microbial populations and the enzymes encoded by these populations within selected samples, has commenced and will be fully reported at a later date.

Figure 4.45. Amplification products resulting from the 2nd PCR of the *Synergistes* genus-specific, nested PCR assay method of McSweeney et al. (2019), size-separated by Agarose gel electrophoresis. Lanes 1, 18, 19,27 are molecular weight size markers (1kb DNA Ladder, GeneRuler). Positive control samples are marked (+ve, *S. jonesii* 10^3 cells/mL; Lanes 2 and 24). A Negative PCR no template control is also marked (Neg, Lane 23). PCR products are in Lanes 3. Site K animal 1 gDNA; 4. Site K animal 1 cDNA; 5. Site K animal 2 gDNA; 6. Site K animal 2 cDNA; 7. Site F animal 6 gDNA; 8. Site F animal 6 cDNA (RF pellet); 9. Site F animal 6 cDNA (RNA-protected pellet); 10. Site F animal 7 gDNA; 11. Site F animal 7 cDNA; 12. Fermentation 17 day 16 gDNA; 13. Fermentation 17 day 16 cDNA (fermentation pellet); 14. Fermentation 17 day 16 cDNA (RNA-protected pellet); 15. Site N animal 1 gDNA; 16. Site N animal 1 cDNA (rumen fluid pellet); 17. Site N animal 1 cDNA (RNA-protected pellet); 20. Site B animal 2 gDNA; 21. Site B animal 2 cDNA (RF pellet); 22.Site B animal 2 cDNA (RNA-protected pellet); 25. Fermentation 17 day 16 cDNA; 26. Site N animal 1 cDNA.

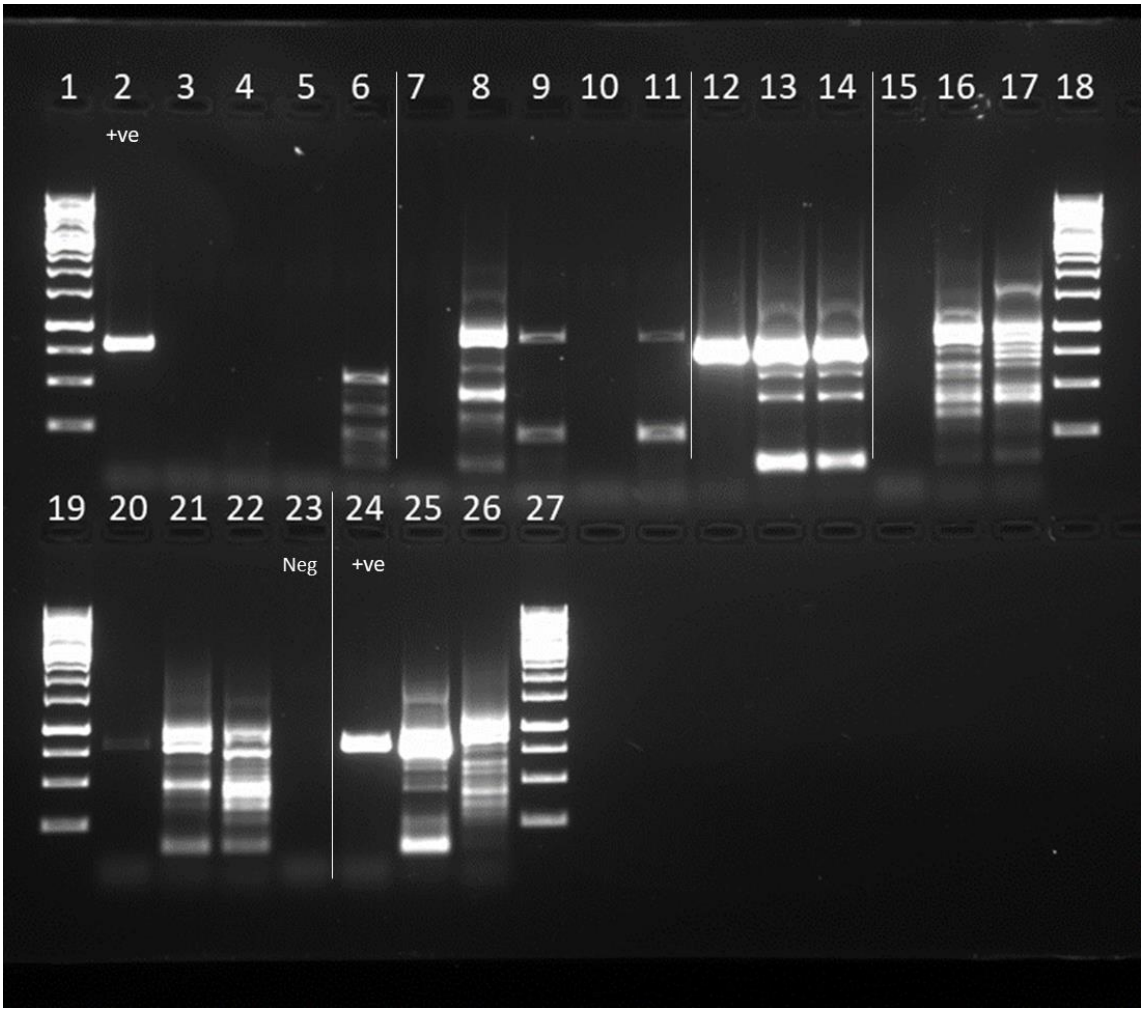


Table 4.8. Summary of toxin degradation results from assays of rumen fluid collected from cattle at property survey sites. Treatment groups: 1 (Never inoculated, grazing Leucaena); 3 (DAF inoculum used or used previously and animals managed, grazing Leucaena) and 4 (Naïve cattle; never grazing Leucaena). Toxin degradation has been categorised as follows - Complete (>90% degradation); Partial High (< 90% and >60% degradation); Partial Low (<60% and > 10% degradation); and None (< 10% degradation).

			Degradation after 48 h Incubation (n)			Degradation after 168 h Incubation (n)		
Site ID	**Treatment Group**	**Cattle Breed and Class (n)**	**Mimosine**	**3,4 DHP**	**2,3 DHP**	**Mimosine**	**3,4 DHP**	**2,3 DHP**
A	4	Holstein Friesian steer (4)	Complete* (4)	None† (4)	None (4)	Complete (4)	None (4)	Complete (1) Partial Low (2) None (1)
B	1	Brahman cow (1), Santa cross steer (1), Droughtmaster cross heifer (1), Charbray cross steer (1)	Complete (4)	Complete (2); Partial High† (2)	Complete (4)	Complete (4)	Complete (4)	Complete (4)
C	3	Brahman cross cow (1), Charolais cross steer (1), cow (1), heifer (1), Droughtmaster cross cow (1)	Complete (5)	Complete (3) Partial High (2)	Complete (5)	Complete (5)	Complete (5)	Complete (5)
D	4	Droughtmaster composite steer (5)	Complete (5)	None (5)	None (5)	Complete (5)	Partial Low (1) None (4)	Complete (4); Partial Low (1)
E	3	Droughtmaster steer (4)	Complete (5)	Partial Low§ (4)	Complete (4)	Complete (4)	Complete (1) Partial Low (3)	Complete (4)
F	3	Droughtmaster bull (6), cow (3), steer (1)	Complete (10)	Complete (3) Partial High (7)	Complete (10)	Complete (10)	Complete (3) Partial High (7)	Complete (10)
G	3	Santa cross steer (2), Murray Grey cross steer (1) Angus steer (2)	Complete (5)	Complete (3) Partial High (2)	Complete (5)	Complete (5)	Complete (3) Partial High (2)	Complete (5)
H	4	Brangus cow (4)	Complete (4)	None (4)	None (4)	Complete (4)	Partial Low (2) None (2)	Partial Low (1) None (3)
I	3	Droughtmaster cross heifer (2), steer (2), Charolais cross heifer (1)	Complete (5)	Complete (5)	Complete (5)	Complete (5)	Complete (5)	Complete (5)
J	3	Wagyu steer (5)	Complete (5)	Complete (5)	Complete (5)	Complete (5)	Complete (5)	Complete (5)

			Degradation after 48 h Incubation (n)			Degradation after 168 h Incubation (n)		
Site ID	**Treatment Group**	**Cattle Breed and Class (n)**	**Mimosine**	**3,4 DHP**	**2,3 DHP**	**Mimosine**	**3,4 DHP**	**2,3 DHP**
K	1	Brahman cross cow (1), Charolais cross cow (1), heifer (1), steer (1), Droughtmaster cross cow (1)	Complete (5)	Partial Low (4) None (1)	Complete (4); Partial High (1)	Complete (5)	Complete (5)	Complete (5)
L	4	Angus heifer (5)	Complete (5)	None (5)	None (3)[#]	Complete (5)	Complete (1) Partial Low (2) None (2)	Complete (2); Partial High (1)
M	1	Angus cow (5)	Complete (5)	Partial High (1) Partial Low (4)	Complete (5)	Complete (5)	Complete (5)	Complete (5)
N	3	Angus cross heifer (2), steer (3)	Complete (5)	Complete (1) Partial High (4)	Complete (5)	Complete (5)	Complete (1); Partial High (4)	Complete (5)

*Complete is >90% degraded; †None is <10% degraded; ‡Partial High is <90%>60% degraded; §Partial Low is <60%>10% degraded.
[#]Animal 1 & 2 received 3,4 DHP instead of 2,3 DHP in the 2,3 DHP degradation assay
(n) = number of animals

4.3 Potential distribution and economic benefits of cultivated Leucaena in northern Australia

This work examined the potential extent and economic benefit of cultivated Leucaena (*Leucaena leucocephala* ssp. *glabrata*) in northern Australia. We used expert knowledge and existing spatial data sets to map potential distribution in Queensland, the Northern Territory, and northern Western Australia. We then conducted an economic analysis to estimate the economic benefit of further adoption across the study area. The work incorporated separate analyses for a new psyllid tolerant cultivar (cv Redlands) and all other commercially cultivated cultivars. A summary of the results is provided below. The work is described in detail in the accompanying report, *The potential distribution and economic benefits of cultivated Leucaena in northern Australia*.

We estimated that up to 27.3M hectares of land in Northern Australia could viably support Leucaena-grass pasture grazing systems. This includes 4.6M hectares in humid coastal areas of Queensland that are suitable for the Redlands cultivar, and where other cultivars have previously been non-viable due to predation from psyllids. The economic analysis suggests a total benefit of $61-123M over the next 40 years across northern Australia. More than 90% of this will be generated in Queensland including $13-26M from cv Redlands cultivation. The primary driver of these economic benefits is the area brought into production, which was estimated at 185,000 hectares over forty years in this work.

Available area was not a limiting factor for expanded adoption of Leucaena-grass systems in northern Australia. Expansion will depend instead on multiple factors including local regulations around issues such as vegetation management and weed Leucaena control, competing land uses, and technical support to ensure better placement, establishment and productivity. This finding applies to all commercial cultivars including cv Redlands.

The abundance of potentially available land presents two opportunities at industry level. Firstly, there is scope for more carefully targeted extension and promotion to develop Leucaena in higher viability areas and ensure better establishment and greater long-term productivity. Secondly, with ample space for Leucaena cultivation there is also room for diligent adherence to the industry code of practice, other local guidelines for weed Leucaena control, and adoption of a sterile cultivar. These are opportunities for better profitability and demonstration of stewardship.

Full results and their discussion are provided in detail in the accompanying report, *The potential distribution and economic benefits of cultivated Leucaena in northern Australia*.

5. Conclusion

5.1 Efficacy of the current and modified Leucaena inoculum

This work clearly showed in 30-day *in vitro* fermentations that the bacterial populations present in DAF Leucaena inoculum initially were negatively affected when fed leaf from either Redlands or Wondergraze cultivars. In particular, the populations of *S. jonesii* decreased and the ability to breakdown the toxins 3,4 DHP and 2,3 DHP were compromised during the first 10 days of the fermentation. This initial negative impact has implications for the ability of the current DAF inoculum's microbial populations to establish when drenched into the rumen of animals grazing these psyllid resistant Leucaena cultivars.

However, microbiome analyses also showed the adaptation over the 30 days of the microbial populations present in the *in vitro* fermenter to efficiently break down the three Leucaena varieties, Cunningham, Redlands and Wondergraze. While the microbiome sequence data results did not always correspond to the results obtained from the qPCR assays, populations of the toxin-degrading bacteria *S. jonesii*, were found to have recovered and increased in the later days of all the fermentations. In addition, in fermentations of all the Leucaena cultivars tested, throughout the fermentation the microbial populations were found to establish, a highly diverse "core" microbial community which could effectively break down the toxins mimosine, 2,3-DHP and 3,4-DHP.

Whilst the bacterial populations did adapt with 'Redlands-Adapted' and 'Wondergraze-Adapted' inoculums produced for use in the CSIRO animal trials it is not economically viable to produce cultivar-specific Leucaena inoculums for the industry. Instead, improvements were investigated to improve the efficacy of the DAF Leucaena inoculum. A multi-variety fermentation (TriMix) was undertaken, which started with three mixed microbial inoculums (Cunningham, Wondergraze-adapted and Redlands-adapted) and fed leaf from all three cultivars. The microbial populations present in the TriMix fermentation were able to completely degrade all three toxins and *S. jonesii* populations were present above 10^6 cells/mL by day 14. Importantly, the *S. jonesii* populations present in TriMix, when fed leaf from a single cultivar immediately increased in number from day 2 onwards and degradation assays showed all three toxins were being degraded from day 10 onwards. Production of the DAF Leucaena inoculum will be modified to the TriMix to ensure the ability of the microbes to establish and use a variety of Leucaena cultivars.

From the sequence dataset we obtained from the CSIRO animal pen trial, however, we could not monitor how the new, cultivar-specific microbial inoculums established in the rumen of cattle fed different levels of Redlands or Wondergraze. This was a consequence of insufficient sequencing depth, and the *S. jonesii* qPCR assay may be required to more accurately monitor how the new, cultivar-specific microbial inoculums become established in animals. In addition, the animal trial had a complex experimental design, tailored to determine the effects of feeding the new Leucaena cultivars on enteric methane production. A more specific and simple feeding trial may be required to better monitor how the microbes contained in the fermenter-produced inoculum persist and assist in the rumen breakdown of the new Leucaena cultivars and the toxins associated with feeding these cultivars.

5.2 On-property survey for the prevalence of rumen detoxification of Leucaena toxins

The results from the on-property survey, which sampled 72 animals from three of the four Treatment groups, indicate that cattle that have not been exposed to Leucaena do not naturally possess rumen bacteria capable of degrading the toxins associated with Leucaena. In the animals sampled in Treatment 1, which had not been inoculated and were grazing Leucaena, the slower

degradation of the 3,4 DHP toxin suggests there may be benefits to inoculating with the DAF Leucaena inoculum to introduce a mix of rumen bacteria including *S. jonesii* which are able to completely degrade the toxins within 48 h.

On Treatment 3 properties where animals were inoculated in the past with the DAF Leucaena inoculum, the management practices used to ensure transfer of the rumen bacteria to new animals were successful in maintaining the between animal transfers in most enterprises. This transfer was demonstrated by animals possessing rumen bacterial capable of degrading all three toxins but were not previously inoculated. On one Treatment 3 property, which managed the transfer by mixing previously inoculated cull cows with new steers, the rumen samples from the five steers all had high levels of the toxic metabolite 3,4 DHP present and did not have rumen bacteria effectively degrading it. This indicated that the steers may not have been sufficiently interacting with the cull cows, to enable the between-animal transfer of the toxin-degrading rumen bacteria and could be at risk of poisoning. We recommended that the steers receive the DAF Leucaena inoculum to ensure they could effectively detoxify 3,4 DHP.

The results from the animals sampled from the Treatment 4 sites demonstrate that cattle, which have not had exposure to Leucaena, do not naturally possess bacteria capable of efficiently degrading the toxic metabolites 3,4 DHP and 2,3 DHP associated with Leucaena. These animals would benefit from receiving the DAF inoculum if they are entering a Leucaena-grass grazing production system.

5.3 Potential distribution and economic benefits of cultivated Leucaena in northern Australia

The potential distribution of Leucaena-grass systems in Queensland, the Northern Territory and northern Western Australia was mapped using expert knowledge and existing spatial data sets. In northern Australia it was estimated that there is up to 27.3M hectares of land that could viably support Leucaena-grass pasture grazing systems. This includes 4.6M hectares in humid coastal areas of Queensland that are suitable for the Redlands cultivar, and where other cultivars have previously been non-viable due to predation from psyllids.

The economic analysis suggested a total benefit of $61-123M over the next 40 years across northern Australia. More than 90% of this will be generated in Queensland including $13-26M from cv Redlands cultivation. The primary driver of these economic benefits is the area brought into production, which was estimated at 185,000 hectares over forty years in this work.

Available area is not a limiting factor for expanded adoption of Leucaena-grass systems in northern Australia. Expansion will depend instead on multiple factors including local regulations around issues such as vegetation management and weed Leucaena control, competing land uses, and technical support to ensure better placement, establishment and productivity. This finding applies to all commercial cultivars including cv Redlands.

The abundance of potentially available land presents two opportunities at industry level. Firstly, there is scope for more carefully targeted extension and promotion to develop Leucaena in higher viability areas and ensure better establishment and greater long-term productivity. Secondly, with ample space for Leucaena cultivation there is also room for diligent adherence to the industry code of practice, other local guidelines for weed Leucaena control, and adoption of a sterile cultivar. These are opportunities for better profitability and demonstration of stewardship.

The results of this work indicates the broader areas of northern Australia where industry can scale up planning with agronomic and economic advisors for best results. This planning could focus on optimal placement of Leucaena at property scale, appropriate weed Leucaena control, and more targeted analysis of potential financial outcomes.

Full results and their discussion are provided in detail in the accompanying report*, The potential distribution and economic benefits of cultivated Leucaena in northern Australia*.

5.4 Project Data Storage Locations

The data generated in the project has been archived and stored as follows -

• Original raw sequence data and the quality filtered data supplied by CSIRO to DAF and sequence data has been archived on the EcoSciences Precinct DAF server and Department of Environment and Science (DES) High Performance Computers (Athena and Apollo).

• Molecular data including PCR assays and quantitative PCR results, and HPLC analyses have been archived on the EcoSciences Precinct DAF server.

• GIS and spatial analyses have been archived on the Rockhampton DAF server.

6. Key findings

The key findings of the project were:

- The psyllid resistant Redlands Leucaena cultivar was found to negatively impact the DAF Leucaena inoculum's ability to degrade two of the toxins, 3,4 DHP and 2,3 DHP, within initial *in vitro* fermentations. Fermenter bacterial populations were then able to be adapted to Redlands as well as the psyllid tolerant Wondergraze cultivar. A TriMix Leucaena inoculum has been developed containing bacterial populations adapted to effectively ferment and detoxify plant material from all three different Leucaena cultivars (Cunningham, Redlands and Wondergraze).
- The property survey results have shown that populations of rumen bacteria able to completely degrade the toxic compounds in Leucaena, are not naturally present in Australian cattle. The rumen bacterial populations in cattle that have never been exposed to Leucaena were unable to degrade the Leucaena toxic metabolites 3,4 DHP or 2,3 DHP. Use of the DAF inoculum to introduce the toxin-degrading bacteria to naïve cattle is recommended to ensure the maximum benefit from utilising Leucaena-pasture grazing. The on-property survey results demonstrated that detoxifying rumen bacteria can be maintained by management practices to ensure between-animal transfer to new cattle being introduced to Leucaena-pasture grazing.
- Mapping estimated up to 27.3M hectares of land in Northern Australia could viably support Leucaena-grass pasture grazing systems. This includes 4.6M hectares in humid coastal areas of Queensland that are suitable for the Redlands cultivar, and where other cultivars have previously been non-viable due to predation from psyllids. The economic analysis suggests a total benefit of $61-123M over the next 40 years across northern Australia. More than 90 % of this will be generated in Queensland including $13-26M from cv Redlands cultivation.

7. Benefits to industry

The development and release of new psyllid resistant Leucaena cultivars offer the potential for increasing uptake by the industry, especially in psyllid prone and high humidity areas. The identification and mapping of potential land areas suitable for Leucaena-grass in this project identified 4.6M hectares in humid coastal areas of Queensland that are suitable for the Redlands cultivar, and where other cultivars have previously been non-viable due to predation from psyllids. The development of a new mixed bacterial rumen inoculum (TriMix) capable of degrading 3,4 DHP and 2,3 DHP efficiently and effectively, when used in animals grazing different Leucaena cultivars,

including the psyllid resistant Redlands cultivar will ensure maximum benefits are gained from animals grazing different cultivars used Leucaena-grass systems.

Industry can utilise the identification and mapping of land areas in Northern Australia which could viably support Leucaena-grass pasture grazing systems and the estimated benefits of future expansions to the industry modelled over the next 40 years to make informed decisions when planning future expansions.

8. Future research and recommendations

8.1 Efficacy of the current and modified Leucaena inoculum

The work in this project identified potential deficiencies of the current DAF Leucaena inoculum which may result in incomplete toxin degradation if it was used in cattle grazing the Redlands or Wondergraze cultivars. An improved mixed bacterial rumen TriMix inoculum was successfully developed, providing bacterial populations adapted to three cultivars which quickly established detoxifying populations in fermentations. The recommendation is that the production of the DAF Leucaena inoculum shift to the TriMix from the next production run.

The on-property survey results to date indicate that the recommendation will be to rumen drench with the DAF Leucaena inoculum to provide cattle grazing Leucaena with rumen bacteria that can completely degrade all the toxins.

The research outcomes of this work were presented as talks at the Australian Association of Animal Sciences (AAAS) conference in Cairns 4-6 July 2022.

8.2 Potential distribution and economic benefits of cultivated Leucaena in northern Australia

There are a number of avenues for future work in this area as the mapping and analysis done in this project does not provide sufficiently fine scaled analysis for use at farm scale to identify suitable areas for Leucaena. It does however suggest broader areas that would be useful starting points for finer scaled planning involving agronomic and economic advisors. Future projects with extension-based activities focussing on the optimal placement of Leucaena at property scale, appropriate weed control and more targeted analysis of potential financial outcomes to demonstrate the benefits of Leucaena for Northern Australian producers should be undertaken.

9. References

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11 Communications

It is planned to present the research outcomes of this project to producers via forums such as FutureBeef or the Leucaena network.

Ouwerkerk, D (2018) Overview of MLA Feeding Leucaena to manage the rumen for maximum beef profit to delegates from the International Leucaena Conference Laboratory visit.

Project data was presented as a poster at the third International Tropical Agriculture Conference (TROPAG 2019), Brisbane, Australia, 11–13 November 2019 and the abstract published in Proceedings (ISSN 2504-3900) – an open access journal published by MDPI.

Ouwerkerk, D., Maguire, A., Gravel, J., Minchin, C., Gravel M., Klieve, A. and Gilbert R. (2020) Effect of feeding different cultivars of *Leucaena leucocephala* on rumen-based *in vitro* anaerobic fermentations. *Proceedings* **36**, 96.

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Gravel, J., Gilbert, R., Maguire, A., Minchin, C. and Ouwerkerk, D. (2022) Do Queensland cattle possess rumen bacteria capable of degrading Leucaena toxins? Project data was presented as an oral presentation at the 34th Australian Association of Animal Sciences Conference, 5-7 July 2022, Cairns, Queensland.

12 Appendix

12.1 Preparation of anaerobic media

For all media:

1. Add dry components to a 2 L conical flask. Add reverse osmosis water (RO H_2O) to the flask and dissolve dry components by swirling. Add salt solutions A and B, and resazurin. Ensure that the volume of the media is above 1 L by adding excess RO H_2O (~1100 mL final volume works well).
2. Boil under constant flow of 95% CO_2/5% H_2 until the volume has reduced to 1 L. The colour of the solution will normally change from blue to a purple or even bright pink as the pH of the media changes during boiling.
3. Continue to gas the media with a constant flow of 95% CO_2/5% H_2 while it is cooling.
4. Add Volatile Fatty Acid (VFA) solution and Cysteine-HCl to the cooled media.
5. Mix well before dispensing into Wheaton bottles (500 mL/bottle), or other bottle volumes as required, under a constant flow of CO_2/H_2 to maintain anaerobic conditions.
6. Autoclave at 105 °C for 45 minutes.

12.1.1 Preparation of rumen fluid/glycerol (RF/Gly) medium

Table 12.1. Ingredients for RF/Gly medium required to make 1 L of media.

Component	/1,000 mL
Peptone	0.1 g
Yeast Extract	0.1 g
Sodium hydrogen carbonate ($NaHCO_3$)	5.0 g
Glucose	2.0 g
Cellobiose	2.0 g
Distilled H_2O[A]	330 mL+
Salt solution A	165 mL
Salt solution B	165 mL
Rumen fluid base	330 mL
Resazurin	1.0 mL
Add after boiling	
VFA solution	10 mL
Cysteine-HCl	0.22 g

[A]RO H_2O = reverse osmosis water

Preparation Notes:

1. Mix well before dispensing.
2. Gassed glycerol: dispense the appropriate volume of glycerol and bubble CO_2 gas through it for at least 30 minutes before adding the medium.
3. Dispense 50 mL medium into a serum bottle containing 50 mL of gassed glycerol. This makes 100 mL of medium and an equal volume (100 mL) of fermenter fluid is added.
4. Dispense 125 mL of medium into Wheaton bottle containing 125 mL of gassed glycerol (250 mL of FR/Gly medium).
5. Normally used to store bacteria after harvesting in a fermenter run (250 mL of fermenter fluid is added).

12.1.2 Preparation of fermenter starter medium

Table 12.2. Ingredients for fermenter starter medium required to make 1 L of media.

Component	/1,000 mL
Peptone	0.5 g
Yeast extract	0.5 g
Sodium hydrogen carbonate ($NaHCO_3$)	5.0 g
Glucose	0.5 g
Cellobiose	0.5 g
RO H_2O	505 mL+
Salt solution A	165 mL
Salt solution B	165 mL
Rumen fluid base	165 mL
Resazurin	1.0 mL
After boiling add:	
VFA solution	10 mL
Cysteine-HCl	0.22 g

[A]RO H_2O = reverse osmosis water

Preparation Notes:

Mix well before dispensing 500 mL per Wheaton Bottle and autoclave at 105 °C for 45 minutes.

12.1.3 Preparation of fermenter salt solution.

Table 12.3. Ingredients for Fermenter Salts Solution required to make 1 L of media

Component	/1,000 mL
Peptone	0.1 g
Yeast extract	0.1 g
Sodium hydrogen carbonate ($NaHCO_3$)	5.0 g
RO H_2O[A]	670 mL +
Salt solution A	165 mL
Salt solution B	165 mL
0.1% w/v Resazurin	1.0 mL
After boiling, add:	
VFA solution	10 mL
Cysteine-HCl	0.22

[A]RO H_2O = reverse osmosis water

Preparation Notes:

Mix well before dispensing 500 mL per Wheaton Bottle and autoclave at 105 °C for 45 minutes.

12.2 Property Survey rumen fluid sample analysis – HPLC SOP

Purpose

The purpose of this procedure is to describe the actions and practices to be taken to analyse the spiked cattle rumen fluid samples and standards from the Property Survey Leucaena field collections for the breakdown of Mimosine to 3-,4-Dihydroxy-pyridine to 2,3-Dihydroxy pyridine via HPLC.

Application/scope

This procedure covers the requirements for sample analysis of spiked cattle rumen fluid (RF) samples by MEG staff via HPLC.

RF samples are collected from cattle – as part of a Leucaena Property Survey within an MLA project. These samples have been taken at set time points (T0, T48 and T168 hours) from up to five animals from each property. The 0.8 mL samples are collected into 1.5 mL microfuge tubes and frozen at -20°C.

The RF samples are spun and filtered to remove particulate material prior to HPLC analysis.

Leucaena QC Standards used are diluted 1:20

Resources

General

HPLC grade Methanol
Phosphoric acid
High quality RO water (>18 Wm)
2 propanol (IPA)
2 L measuring cylinder capped
2 L Schott bottle
500ml Schott bottle x2 for methanol and water

Equipment

Waters 2695 separation module
Waters 486 absorbance detector
Phenomenex column 00G-4299-E0 Aqua 5 mm C18 125Å LC column 250 x 4.6 mm
Computer with Waters EmPower software

Prepare the day before HPLC run or on the day, time permitting:

Phosphate buffer mobile phase: Using the 2 L measuring cylinder prepare 2.0 L of 0.1% phosphoric acid (2 mL) plus 1% methanol (20 mL) using deionised RO water (must be greater than 18 Wm quality) (can be filtered through a 0.45 µm filter) and store in 2L Schott bottle at 4 °C. The buffer should be prepared with a minimal amount of aeration of the volume i.e. run the liquids down the side of the cylinder and bottle.
The standards can be prepared, aliquoted and frozen at -20 °C at any time. Using the same 10 mM standards used to spike the RF on property collection (see SOP for RF sample collection from cattle for Property Survey) dilute (1:20 equivalent to 0.5 mM) individually in high purity RO water (must be greater than 18 Wm quality) and aliquot 120 µL into labelled screw top vials with the smaller LVI (150 mL). Seal lid and freeze at -20°C. **Caution note re-freezing**. Loosen lid until frozen to prevent breaking the LVI as liquid freezes.

Prepare the day of HPLC run:

Thaw a set of diluted standards at room temperature, loosen the vial lid to prevent a partial vacuum.
Check and refresh as required:
*Red tube C – fresh RO water
Blue tube B – 100% methanol

*Yellow tube A – phosphate buffer
Green tube – 10% IPA (recycle needle wash and PTFE seal wash)
Cleaning bottle – 50% RO water / 50% methanol solution
*Rinse with RO water and place the fritted tubing in the respective Schott bottle
Power on 2695 separation module and UV468 (warm up 10 minutes)
(*If 'FAIL 1' appears on the detector the battery needs replacing see page 110 of Operator's Manual. Once replaced turn on the detector and wait for the CAL to be completed then **diag** enter **13 Enter** and add the IEEE-488 address **20 Enter** the detector then must be turned off for 3 minutes and then power on)

Instrument Setup:

At instrument screen click "**Menu / status**" to bring up control screen
Set composition to 100% B (methanol blue tube)
Wet prime mobile phase lines.
Direct Function key
Select **Wet Prime** and set flow rate to 7 mL/ min for 10 minutes **OK**
Allow line B to prime for 2 to 2:30 minutes
Change composition to 100% C (RO water)
Allow line C to prime for 2 to 2:30 minutes
Change composition to 100% A (Phosphate buffer) and repeat
Change composition to 100% C for about 1 minute.
Once primed **Stop Prime** and **Abort Prime** or can allow time to run out
Set composition to 100% B at 0.5 mL/minute through the column for approximately 10 minutes.

Condition the column:

Set the column temperature to 40 °C in the **SET** block.
Set composition to 50% B and 50% C flow rate 0.5 mL / min.
Allow time to stabilise the pressure (± 50psi variation is acceptable) and make a note of the pressure. (approx.1400 psi)
inconsistent pressure can be due to check-valves failing or a leak in the system
increased pressure from previous runs can be due to build-up of particles on the Guard column and eventually blockage of the column.

Sample Loading:

Turn on the computer. (**administrator**; **Ari** computerID)
Once the instrument has been setup and conditioned, loosen all the lids (not necessary with pre-split) and load the vials onto the carousels in order and position in the instrument. Position the standards in order mimosine, 3,4 DHP and 2,3 DHP in positions A1, 2 and 3.
Start EmPower software (**MEORG**) **Enter**
Select **Browse Projects** – select mimosine DHP and OK
On window select **Run Samples** (blue cylinder action button)
In the instrument **UV95 Mimosine DHP** window – **Instrument Method** (bottom right drop down).
Select **Test Std Mimosine** (this will select the method to run the 3 standards to check the run times for each (currently at approximately 3; 4 and 10 minutes)).
Select **Setup** (this will set the flow rate to initial conditions, 1 mL/minute; 100% A; zero the detector and set the column temperature). Allow to stabilise for at least 10 minutes before commencing **Run mode:**
On the **Sample** tab click on **Load** button to  load sample names from Leucaena template.
Check the runtime still covers the 2,3 DHP peak time (12 minutes)
Run Sample Set and rename sample run ensure run time is adequate (12 minutes)
Select **Run only / continue on fault**
Select run **Green** action button
If the standards are within the acceptable range, queue up the full **Mimosine Method Set** using the **Leucaena Template Sample** Set to follow on from **Test Std Mimosine** (Providing there are no major

changes in the run time required). Go to the **Sample tab** and **load samples** from the template (check the sample run time is OK for the current retention times based on the Test Stds results. This time will decrease over time as the column ages). Run the sample set **green button**, the list will go across to the **Sample Set** tab and be queued under the Test Stds run.
Check the required volume of Phosphate buffer A (usually approximately 2L). If necessary, top up immediately.
Note down the retention times in the front of the workbook
Shutdown after auto-flush completed:
Using the computer controlling software (EmPower) turn the pump back on to flush the lines further. From **Run Sample screen** select **D** button, bottom of screen and set flow rate (minimum 0.25 mL / minute maximum 0.5 mL / minute) use a composition of 50% B and 50% C mobile phase so that if there are any samples to be rerun the column is still ready. Allow to run while reviewing data.
Review of Chromatograms
Minimise **Run Sample** window and go to the **Sample Sets** tab (if the set isn't listed **Update** to refresh list).
Highlight the set of data to be reviewed click **Review** action button 1st on bar or 'right click' **Review** in the dropdown menu.
File Open select the Method Set **Mimosine** (the Test Std Mimosine is not required as this was just to check run times in the Method set were sufficient).
Set the scale by right clicking the chromatogram select **Properties** in menu – **scaling** tab - "**get values from plot**" to remove the auto-scaling. This will keep the peaks all to the same scale during review.
Set up the **component IDs** for the peaks (2nd row of buttons) using the standards.
Review the components, adjust if necessary.
Review the Integration Events to ensure that none of the shoulders of the peaks of interest have been crossed by Valley to Valley (VV) and or Integration (II).
Check that the retention time window (RTW - blue bracket either side of peak) hasn't moved across the several standards injections at the start, middle and end of run. Try to average the peaks from the actual retention times and check back to the first set of standards to ensure the RTW hasn't moved off the peak and the ID lost.
Review the samples to ensure that the peaks are clean and that the 3,4 DHP peak hasn't merged with the noise ahead of it.
Once chromatograms and Id labels are acceptable **Exit** Review and **Save** Processing Method "**clear curve**".
Process Data:
Highlight the **Sample Set** and Click **Process** "**calculator**" action button (4th from left) use acquisition method set, **calibrate and quantitate**.
Review processed data:
Result Set tab, open the result of interest (update if not visible)
Review each chromatogram to ensure satisfactory integration and quantitation, set the scale as before.
Adjust Integration manually by dragging the event markers as required **SAVE Result**. If there become more than a few samples requiring adjustments, then reintegrate all of the results by returning to **Sample Set** adjust method and reprocess.
Export Data:
Go to **Results Set** and click on **Export** action button (6th on left side) **OK**
On the desktop there is a shortcut button to the folder where the file is exported to.
Open **Export** file and remove the first line of text up to "**Sample Name"** and 'save as' to USB. The file can be imported into the Excel template workbook (**HPLC Leucaena QC data analysis**) via the **data** tab **From Text/CSV** and load the data, this will tabulate the data in a new worksheet. (**REU: \Secure\Projects\Leucaena HPLC data analysis \HPLC Property Survey QC data analysis** (Excel macro enabled template))
Copy and paste the sample data only, from the imported data to the **Calculator** tab designated paste region, this will autofill the **PrePivot data** table in the correct format. Open the **Pivot table** tab and

click on the refresh button to the right of the Pivot table, this will bring in the data and prepare the results chart.

Cleanout / Shutdown of instrument:

NB the closing of the **Run Sample close** on the computer will release the instrument from computer remote control.

At this point it is worth reviewing the data in Excel to ensure there are no samples requiring rerunning before the instrument is fully shutdown.

Set flow to 0 mL/min

Remove lines A (Phosphate buffer) and C (RO water) wash and place lines into the Cleaning bottle (50% methanol 50% water).

To flush the lines **Set composition to 100% A** Wet prime mobile phase lines –

Direct Function key

Select **Wet Prime** and set flow rate to 7 mL/ min for 10 minutes **OK**

After 2 to 3 minutes change **composition to 100% C**

After 2 to 3 minutes change **composition to 100% D** (this just to keep the D line filled)

After 1 minute set **composition to for 4 lines to 25%** to rotate the switching valve through for a flush clean.

After 30 seconds to a minute **Stop Abort Prime**.

Open door and remove samples from carousels, the outer vials can be reused, discard the LVIs and lids.

Power off for Separator and Detector.

Empty the waste carboy.

Related and reference documents

Guide	HPLC Trouble-shooting Technical Notes Phenomenex
Manual	Waters 2695 LC separation module & Waters 486 absorbance detector
Standard Operating Procedure	Property Survey RF sample prep for HPLC

12.3 Stomach tubing of cattle SOP

Microbial Ecology Group
Standard Operating Procedure

Use of stomach tube for the collection of rumen fluid in cattle

Policy: This procedure may only be performed by, or under the supervision of operators skilled in the technique identified in active Animal Ethics Approvals to collect rumen fluid.

Precautions: Restraint of the animal's head is required for the procedure. It is preferable to have the animal in a crush with its head restrained in the head bail. Regurgitation and aspiration of rumen fluid into the lungs may occur if the animal is forced to elevate its head. The presence of horns often requires modification of the procedure to ensure operator safety. Stomach tubes should be checked after use to ensure all surfaces are smooth.

Equipment: Stomach tube (approx.1.5 – 2 m of reinforced tubing with stainless steel end piece with rounded end and holes)

Stainless steel gag (with side grip and rounded edges)

Plastic side arm flask (2 L volume) with rubber bung that fits stomach tubing and tubing that fits side arm and long enough to go to hand pump

Hand pump (e.g. Wanderer Large Dual Action Hand Pump)

Plastic Buckets (2 or 3 set up to rinse gag, stomach tubing and 2 L flask between animals)

Procedure: Check the hand pump is connected to deflate (pull a vacuum) and connect the tubing from the pump to the side arm of the 2 L plastic flask. Place the rubber bung into the top opening of the flask and insert the non-bevelled end of the stomach tubing through the hole. Place a hand around the holes in the end of the stomach tube and pump a couple of times to ensure you can feel a vacuum being pulled.

The size and disposition of the animal will determine if an assistant is required to restrain the animal and open its mouth to place the gag across the tongue. The stomach tube is inserted through the gag and down the back of the throat, down the oesophagus into the rumen.

Once the tube is gently inserted, working as a team one person commences slow hand pumping to create a vacuum whilst the other person moves the stomach tubing slightly back and forth in the rumen. Fluid should come up the tube and into the 2 L flask. Do not collect more than 1.5 L into the flask.

Withdraw the tubing with the animal's head as low as possible to minimise the risk of accidental aspiration of rumen content. Once the tube is removed, remove the gag and restraint of the animal's head.

Between animals - rinse gag, stomach tubing (inside and out) and 2 L side arm flask in sequential buckets of water if a tap is not available.

Version: 2
Modified by: Diane Ouwerkerk
Date Modified: 04 August 2021 1

12.4 Core fermenter populations at later days of the fermentations (≥ 10 days)

Table 12.4. Taxonomic classification of fermenter bacterial and archaeal populations designated as shared core microbial communities present in the later days (≥ 10 days) of fermentations supplied with one of the three Leucaena cultivars tested (either Cunningham, Redlands or Wondergraze). Taxonomic classification levels as designated using the SILVA database (version 132) include D_0, Domain; D_1, Kingdom; D_2, Phylum; D_3, Class; D_4, Order; D_5, Family; D_6, Genus; D_7, Species.

41 common elements identified in fermentations of either Cunningham, Redlands or Wondergraze leaf material
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotella 1;__
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Rikenellaceae;D_5__Rikenellaceae RC9 gut group;__
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;__;__
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotella 1;D_6__Prevotella brevis
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Butyrivibrio 2;__
D_0__Bacteria;D_1__Spirochaetes;D_2__Spirochaetia;D_3__Spirochaetales;D_4__Spirochaetaceae;D_5__Treponema 2;__
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnospiraceae FE2018 group;D_6__uncultured bacterium
D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Desulfovibrionales;D_4__Desulfovibrionaceae;D_5__Desulfovibrio;__
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Rikenellaceae;D_5__Rikenellaceae RC9 gut group;D_6__unidentified rumen bacterium RF36
D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Selenomonadales;D_4__Veillonellaceae;D_5__Anaerovibrio;__
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Christensenellaceae;__;__
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Rikenellaceae;D_5__SP3-e08;D_6__uncultured bacterium
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Oribacterium;__
D_0__Bacteria;D_1__Tenericutes;D_2__Mollicutes;D_3__Anaeroplasmatales;D_4__Anaeroplasmataceae;D_5__Anaeroplasma;D_6__Anaeroplasma abactoclasticum
D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Selenomonadales;D_4__Veillonellaceae;D_5__Schwartzia;__
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;__;__;__
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Eubacterium] ventriosum group;D_6__Lachnospiraceae bacterium RM5
D_0__Bacteria;D_1__Tenericutes;D_2__Mollicutes;D_3__Mollicutes RF39;__;__;__
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-005;D_6__uncultured rumen bacterium
D_0__Bacteria;D_1__Firmicutes;D_2__Negativicutes;D_3__Selenomonadales;D_4__Acidaminococcaceae;D_5__Succiniclasticum;__
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-002;__
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotellaceae NK3B31 group;D_6__rumen bacterium NK3B31
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Eubacterium] ruminantium group;__
D_0__Bacteria;D_1__Cyanobacteria;D_2__Oxyphotobacteria;D_3__Chloroplast;__;__;__
D_0__Bacteria;D_1__Tenericutes;D_2__Mollicutes;D_3__Anaeroplasmatales;D_4__Anaeroplasmataceae;D_5__Anaeroplasma;__
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Pseudobutyrivibrio;__
D_0__Bacteria;D_1__Spirochaetes;D_2__Spirochaetia;D_3__Spirochaetales;D_4__Spirochaetaceae;D_5__Treponema 2;D_6__uncultured bacterium
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__p-251-o5;D_5__uncultured bacterium;D_6__uncultured bacterium
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae NK4A214 group;__
D_0__Archaea;D_1__Euryarchaeota;D_2__Methanobacteria;D_3__Methanobacteriales;D_4__Methanobacteriaceae;D_5__Methanosphaera;__
D_0__Bacteria;D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Streptococcus;__

D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcaceae UCG-014;__
D_0__Bacteria;D_1__Proteobacteria;D_2__Deltaproteobacteria;D_3__Desulfovibrionales;D_4__Desulfovibrionaceae;D_5__Bilophila;D_6__uncultured rumen bacterium
D_0__Bacteria;D_1__Synergistetes;D_2__Synergistia;D_3__Synergistales;D_4__Synergistaceae;D_5__Pyramidobacter;__
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__p-2534-18B5 gut group;__;__
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococcus 2;__
D_0__Bacteria;D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Aeromonadales;D_4__Succinivibrionaceae;D_5__Anaerobiospirillum;D_6__uncultured bacterium
D_0__Bacteria;D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Kandleria;D_6__Kandleria vitulina
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Eubacteriaceae;D_5__Eubacterium;__
D_0__Bacteria;D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;__;__
D_0__Bacteria;D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Family XIII;D_5__Anaerovorax;D_6__uncultured rumen bacterium

12.5 Animal Ethics Approval

Queensland Government	*DAF Animal Ethics*	Form: AE 07
	DECISION of the ANIMAL ETHICS COMMITTEE (AEC)	

1. Applicant (person submitting the application) details

Name: Diane Ouwerkerk		
Organisation: DAF		Centre: Ecosciences Precinct
Postal Address: Level 2A East, 41 Boggo Road Dutton Park Qld 4102		
Phone: 3708 8391	Mobile: 0407 022 470	E-Mail: diane.ouwerkerk@daf.qld.gov.au
Registered person:	DAF	

2. Project Details

Title of the Project	AEC Application Reference Number
MLA B.GBP.0026 'Feeding Leucaena to manage the rumen for maximum beef profit', On property survey of prevalence of rumen detoxification of Leucaena toxins.	**SA 2021/08/795**

3. AEC Decision

The project application has been considered by the AEC and is:

Approved

Any inquiry regarding this response should be directed to the AEC Coordinator or Chair in the first instance. The Coordinator or Chair may be contacted via the DAF Call Centre on 13 25 23.

Purpose:	The Maintenance and Improvement of Human or Animal Health and Welfare
Category:	Minor conscious intervention without anaesthesia
Activity location:	Various locations

Comments:

Please note the use of 120 animals has been approved in case extra animals were required.

Period of approval inclusive of the following start and end dates: Approved Start Date: 1 September 2021 Approved End Date: 31 August 2024	Animal type and number approved: Cattle - 120

Important information

1. **This approval** is for that work as approved in this decision and only within the start and end dates unless amended by a subsequent AEC decision made in accordance with the requirements of the *Animal Care and Protection Act 2001*, the *Australian code for the care and use of animals for scientific purposes* (refer to 2 b) below).

   Any animal use outside this approval will constitute a breach of Section 91 of the *Animal Care and Protection Act 2001* and is subject to a maximum penalty of 300 penalty units or one year's imprisonment.

   As well as obtaining an AEC approval, a person must not use an animal for a scientific purpose unless the person is registered or retained by a registered person and acting in the course of their retainer.

   Whilst an application can be lodged by an applicant on behalf of a Registered Person, the Registered Person is responsible for, and holds, the approval. The applicant also has responsibilities under the Code and will be ultimately responsible for the care and use of animals in the project unless otherwise stated.

   Unless otherwise stated, this approval applies only to work conducted within Queensland.

2. **The AEC requires the Applicant to:**

   a) ensure compliance by all investigators with all conditions set out in this decision in addition to the general requirements of the *Animal Care and Protection Act 2001*, the *Australian code for the care and use of animals for scientific purposes* and all other relevant Commonwealth and State legislation.

   b) submit an Amendment Request (Form AE 08) for any proposed change to a project approval prior to that change being implemented (refer to Procedural Guideline 04);

   c) report any unexpected or adverse event that impacts on the welfare of any animal used in this project (refer to Procedural Guideline 03);

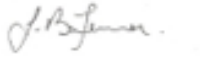
   d) submit Annual Progress Reports (Form AE 10) early each year; and

   e) submit a Project Completion Advice (Form AE 09) upon completion of this project.

3. **Endorsement:** Approval of your project application/amendment request by the AEC is not an endorsement of the project by either the Department of Agriculture and Fisheries or the Queensland Government and is not an endorsement of the Applicant, its products or its processes generally by the AEC, Department of Agriculture and Fisheries or the Queensland Government and no one should assert any such endorsement.

4. **Correspondence:** All correspondence with the AEC in relation to this project should be via email to your AEC contact and cite the name of the Applicant, title of the project and the AEC Application Reference Number.

5. **Grievance:** If the Applicant feels that the AEC has erred in its decision regarding any aspect of the project, the Applicant can submit a complaint (refer to Procedural Guideline 05).

Name of AEC	Staff Access AEC
AEC Address	Ecosciences Precinct 41 Boggo Road Dutton Park Qld 4102
Name of AEC Chair	Lex Turner
Chair contact details	T: 07 3708 8507 M: 0427 001 427 email: lex.turner@daf.qld.gov.au
Signature	
Date of Decision	9 August 2021

12.6 Potential distribution and economic benefits of cultivated Leucaena in northern Australia

'The potential distribution and economic benefits of cultivated Leucaena in northern Australia' is included as a separate document.

The potential distribution and economic benefits of cultivated leucaena in northern Australia

December 2021

Queensland Government

This publication has been compiled by Terry Beutel and Fred Chudleigh of Agriscience Queensland Department of Agriculture and Fisheries,

Contents

Summary

This work examines the potential extent and economic benefit of cultivated leucaena (*Leucaena leucocephala* ssp. *glabrata*) in northern Australia. We used expert knowledge and existing spatial data sets to map potential distribution in Queensland, the Northern Territory, and northern Western Australia. We then conducted an economic analysis to estimate the economic benefit of further adoption across the study area. The work incorporated separate analyses for a new psyllid tolerant cultivar (cv Redlands) and all other commercially cultivated cultivars.

We estimate that up to 27.3M hectares of land in Northern Australia could viably support leucaena-grass pasture grazing systems. This includes 4.6M hectares in humid coastal areas of Queensland that are suitable for the Redlands cultivar, and where other cultivars have previously been non-viable due to predation from psyllids. The economic analysis suggests a total industry benefit of $61-123M over the next 40 years across northern Australia. More than 90% of this will be generated in Queensland including $13-26M from cv Redlands cultivation. The primary driver of these economic benefits is the area brought into production, which was estimated at 185,000 hectares over forty years in this work.

Available area is not a limiting factor for expanded adoption of leucaena-grass systems in northern Australia. Expansion will depend instead on multiple factors including local regulations around issues such as vegetation management and weed leucaena control, competing land uses, and technical support to ensure better placement, establishment and productivity. This finding applies to all commercial cultivars including cv Redlands.

The abundance of potentially available land presents two opportunities at industry level. Firstly, there is scope for more carefully targeted extension and promotion to develop leucaena in higher viability areas and ensure better establishment and greater long-term productivity. Secondly, with ample space for leucaena cultivation there is also room for diligent adherence to the industry code of practice, other local guidelines for weed leucaena control, and adoption of a sterile cultivar. These are opportunities for better profitability and demonstration of stewardship.

This work indicates broader areas of northern Australia where industry can scale up planning with agronomic and economic advisors for best results. This planning could focus on optimal placement of leucaena at property scale, appropriate weed leucaena control, and more targeted analysis of potential financial outcomes.

Introduction

Leucaena (*Leucaena leucocephala*) is a leguminous woody plant used for a number of purposes globally. Leucaena leaves have potentially high nutritional value for cattle, and this has resulted in wide use as a supplementary food for ruminants including cattle. Cultivation of leucaena (primarily *L. leucocephala* ssp. *glabrata*) to augment pastures for beef cattle has seen a steady increase in eastern Australia since the 1970s (Beutel *et al.* 2018).

Leucaena is generally cultivated in widely spaced rows interspersed by native or introduced pasture species, and its use comes with a number of potential challenges and benefits. The challenges include the difficulty and cost of establishment (Shelton and Dalzell 2007, Buck *et al.* 2019) and the associated risks of leucaena proliferating as a weed outside cultivation areas (Campbell *et al*. 2019). The benefits include the potential for positive long term financial benefits for the grazing business (Bowen and Chudleigh 2021a), potential reductions in cattle greenhouse emissions (Harrison *et al.* 2015) and nitrogen fixation (Shelton and Dalzell 2007).

The majority of cultivated leucaena in northern Australia is sown in Queensland where cultivations are largely confined to areas with 600-800mm average annual rainfall. Leucaena in these areas is relatively safe from destructive predation by the introduced psyllid *Heteropsylla cubana* which has limited cultivated leucaena in more mesic areas of the state (Dalzell *et al.* 2006). Psyllids do not yet appear to present the same challenges in other parts of northern Australia (Revell *et al.* 2019, A Cameron *pers. comm*.). Reasons for this are not clear, but as leucaena species are the primary hosts for *H. cubana* (CABI 2021) there may be insufficient leucaena in these jurisdictions to sustain psyllid populations. Other factors such as the generally longer dry season in much of the Northern Territory and Western Australia may also play a role. Whatever the case though, psyllids do not currently appear to significantly challenge leucaena cultivation in these jurisdictions.

Meat and Livestock Australia (MLA), in association with the University of Queensland, commenced work in 2002 to develop psyllid tolerant strains of Leucaena. The Redlands strain was selected from these trials for further development (Dalzell *et al.* 2013, Dalzell 2019) and is now available for commercial use. Redlands' tolerance to psyllids provides significant potential to expand leucaena–grass pasture systems in the Queensland beef industry into higher (>800mm) rainfall areas, and Shelton *et al.* (2017) estimated that it may open up 4.4 - 5.7M hectares of grazing land to leucaena cultivation.

There have been a number of historical efforts to map and/or estimate areas available for Leucaena-grass systems in northern Australia (Burgess 2010, Kenny and Drysdale 2019, Whitsed 2017). This type of mapping can potentially address two separate goals and is largely dependent on the scale of the spatial layers used to map potential distribution. If underlying spatial layers are sufficiently detailed such mapping could identify suitable cultivation sites at enterprise scale as well as more regional scales. Where underlying spatial layers are coarser, mapping would not be suitable at enterprise scale, but could still document broader regional patterns, and inform regional RD&E efforts to assist successful adoption and inform economic analyses.

This study comprises two research activities. The first uses publicly available spatial data and the expert knowledge of RD&E personnel with experience in leucaena cultivation to map and quantify the potential distribution of cultivated leucaena in northern Australia (Queensland, the Northern

Territory and northern Western Australia). The second activity provides an analysis of the likely economic benefits (marginal benefit expressed as amortised net present value (NPV)) of new cultivations in these regions. In both analyses we separated results for the new Redlands cultivar from all other cultivated leucaena cultivars.

Methods

This section describes the methods for both the potential distribution mapping (2.1) and the economic analysis (2.2).

Potential distribution mapping

The project study area includes all grazing land in Queensland, the Northern Territory and Western Australia north of 26°S and covers approximately 2.45M km² (Figure 1).

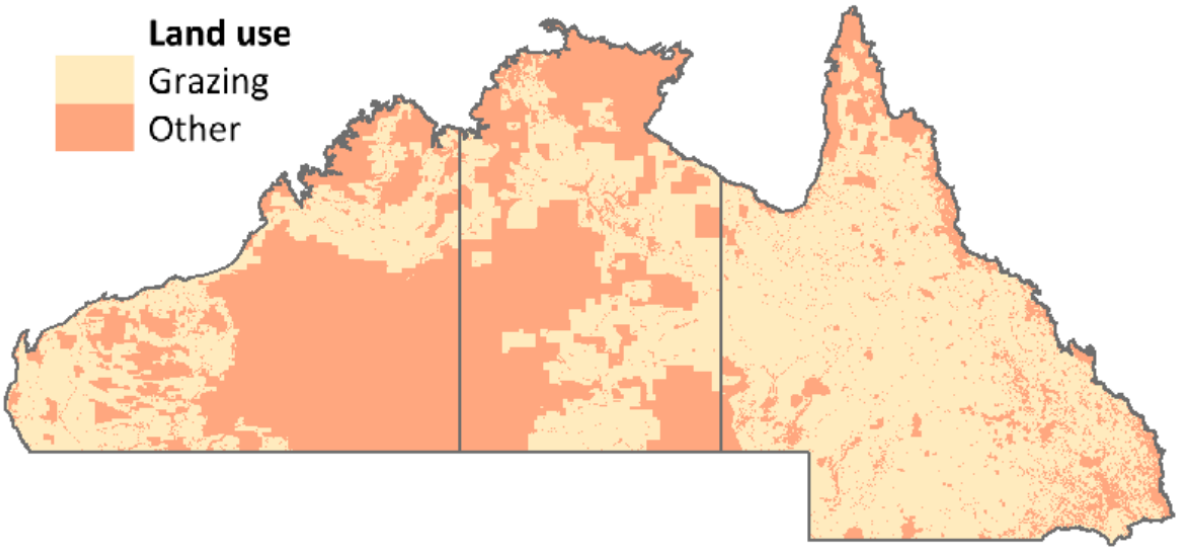


Figure 1. The study area and distribution of grazing land within it.

For the purposes of this work, potential leucaena areas are defined as grazing land where leucaena (Redlands or Other (commercially cultivated varieties of *L. leucocephala ssp. glabrata*)) could be viably cultivated and used to feed cattle in a commercial leucaena – grass pasture system. Leucaena is also an invasive weed species in many jurisdictions (both ssp*. glabrata* and ssp*. leucocephala*) with significant potential for further increase (Walton 2003, Campbell *et al.* 2019). This evaluation does not address the potential for further weed spread, though obviously cultivation has a potential role in weedy leucaena proliferation.

We used a combination of expert knowledge and existing spatial analysis to develop mapping of the potential distribution of leucaena in northern Australia.

1. Through literature review and consultation with five regional experts in leucaena cultivation and management we identified a set of seven spatial layers (Appendix 1) that we used to map potential leucaena distribution. Inclusion of any layer in the mapping process was on the basis of two criteria; the layer was available for the full extent of the study area and the variable mapped

in the layer was identified by members of the expert panel as important to the viability of commercial leucaena – grass pastures.

2. Experts identified potential partitions for the selected layers that divided the layers into three mutually exclusive zones (optimal, viable and non-viable) based on the mapped variable (Table 1). Viable conditions were those where commercially cultivated leucaena was expected to establish and grow successfully. Optimal conditions were not defined clearly during this part of the work but can be considered as those where commercially cultivated leucaena was expected to thrive. It should be noted that the lack of definition for optimal conditions ultimately meant that the final map products only discriminate viable and non-viable land capability, with optimal areas an unquantified subset of viable areas.

3. We used a probabilistic approach to mapping potential distribution. A total of 50,000 random points were distributed across the study area. The value of each map layer (Step 1) at each point was extracted to that point so that each point was attached to a series of layer values relevant to its location.

4. An interactive spreadsheet was developed to assist expert panel members map potential leucaena distribution according to their own experience and knowledge. The spreadsheet incorporated the 50,000 random points and their attached values (Step 3) and the variable partitions (Step 2). Users could select combinations of variables and partitions which interactively adjusted a map and table built into the spreadsheet so that experts could see the distribution of Optimal, Viable and Non-Viable points and the estimated area in each state/territory in each viability class (Figure 2). The overall viability of any point equalled the lowest viability for all layers selected by the expert at that point (Table 2).

5. This tool was distributed to the original panel of five regional experts plus two additional regional experts. All experts supplied the study with their variable and partition selections and the state/territory to which their respective solution applied (Appendix 2). In addition, they were invited to provide any additional assumptions / caveats / concerns they thought relevant (Appendix 3). This was deemed important since all experts expressed some difficulty matching the mapped layers to their expectations.

Table 1. Map layer partitions. Note that the land use layer was partitioned as Non-viable for non-grazing land and Optimal at all other locations. Experts were unable to exclude this variable partition so it is not included in the table.

Variable	Optimal	Viable	Non-viable	Choice
Soil pH to 60cm	6.5 – 8.5	5.5 – 6.4 and 8.6 – 9.0	<5.5 and >9.0	1
	6.0 – 8.5	5.5 – 6.0 and 8.6 – 9.0	<5.5 and >9.0	2
	5.5 – 8.5	8.6 – 9.0	<5.5 and >9.0	3
Available water capacity	>150mm in top 1m	100-149mm in top 1m	<100mm in top 1m	4
	If soil is Kandosol then >150mm in top 1.5m, else as above	If soil is Kandosol then 100-149mm in top 1.5m, else as above	If soil is Kandosol then <100mm in top 1.5m, else as above	5
Annual rainfall (mm)	>700	550-700	<550	6
	>600	550-600	<550	7
	>900	750-900	<750	8
Dry season length (months<20mm)	<6 months	6-7 months	>7 months	9
	<5 months	5-6 months	>6 months	10
	<4 months	4-5 months	>5 months	11
	<3 months	3-4 months	>4 months	12
Soil order	Chromosols, Dermosols, Ferrosols, Kandosols, Vertosols	Sodosols, Calcarosols	Anthroposols, Hydrosols, Kurosols, Organosols, Podosols, Rudosols, Tenosols	13
Slope (%)	<5%	6-10%	>10%	14
Woody cover	0-10%	11-20%	>20%	15
	0-9%	10-14%	>14%	16

6. Expert solutions were aggregated per state/territory. The overall viability of any point was calculated as the proportion of expert scores for that point classed either Viable or Optimal. So, for example if the three Queensland experts respectively classed a point as Viable, Non-viable and Viable, then the point receive a viability score of 0.67.

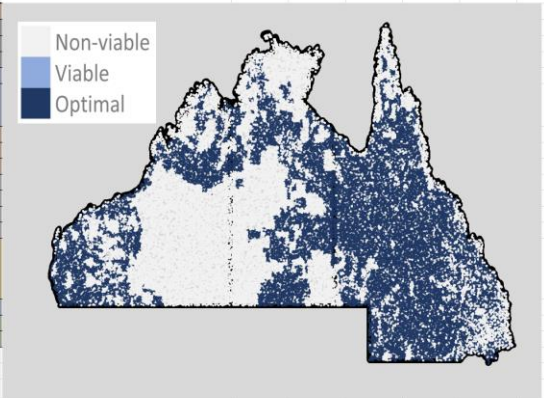
7. The study area was stratified into 14 regions used for the Australian Agricultural Survey (AGSURF 2020) and the area viable for leucaena in any region was calculated as the average viability score of all points in the region multiplied by the area of the region.

8. Viable leucaena areas withing Queensland were further split based on average annual rainfall. Viable areas with >800mm annual rainfall were assigned to Redlands leucaena and all other areas to Other leucaena cultivars.

A

Variable	Optimal	Viable	Non-viable	Choice
Soil pH to 60cm	6.5 - 8.5	5.5 - 6.4 and 8.6 - 9.0	<5.5 and >9.0	
	6.0 - 8.5	5.5 - 6.0 and 8.6 - 9.0	<5.5 and >9.0	
	5.5 - 8.5	8.6 - 9.0	<5.5 and >9.0	
Available water capacity	>150mm in top 1m	100-149mm in top 1m	<100mm in top 1m	
	If soil is Kandosol then >150mm in top 1.5m, else as above	If soil is Kandosol then 100-149mm in top 1.5m, else as above	If soil is Kandosol then <100mm in top 1.5m, else as above	
Annual rainfall (mm)	>700	550-700	<550	
	>600	550-600	<550	
	>900	750-900	<750	
Dry season length (months<20mm)	<6 months	6-7 months	>7 months	
	<5 months	5-6 months	>6 months	
	<4 months	4-5 months	>5 months	
	<3 months	3-4 months	>4 months	
Soil order	Chromosols, Dermosols, Ferrosols, Kandosols, Vertosols	Sodosols, Calcarosols	Anthroposols, Hydrosols, Kurosols, Organosols, Podosols, Rudosols, Tenosols	
Slope (%)	<5%	6-10%	>10%	
Woody cover	0-10%	11-20%	>20%	
	0-9%	10-14%	>14%	

Hectares	Optimal	Viable	Non-viable
WA	48,706,238	0	90,132,062
Qld	139,318,972	0	32,984,328
NT	56,605,134	0	76,834,866



B

Variable	Optimal	Viable	Non-viable	Choice
Soil pH to 60cm	6.5 - 8.5	5.5 - 6.4 and 8.6 - 9.0	<5.5 and >9.0	
	6.0 - 8.5	5.5 - 6.0 and 8.6 - 9.0	<5.5 and >9.0	
	5.5 - 8.5	8.6 - 9.0	<5.5 and >9.0	
Available water capacity	>150mm in top 1m	100-149mm in top 1m	<100mm in top 1m	
	If soil is Kandosol then >150mm in top 1.5m, else as above	If soil is Kandosol then 100-149mm in top 1.5m, else as above	If soil is Kandosol then <100mm in top 1.5m, else as above	
Annual rainfall (mm)	>700	550-700	<550	
	>600	550-600	<550	1
	>900	750-900	<750	
Dry season length (months<20mm)	<6 months	6-7 months	>7 months	
	<5 months	5-6 months	>6 months	
	<4 months	4-5 months	>5 months	
	<3 months	3-4 months	>4 months	
Soil order	Chromosols, Dermosols, Ferrosols, Kandosols, Vertosols	Sodosols, Calcarosols	Anthroposols, Hydrosols, Kurosols, Organosols, Podosols, Rudosols, Tenosols	
Slope (%)	<5%	6-10%	>10%	
Woody cover	0-10%	11-20%	>20%	
	0-9%	10-14%	>14%	

Hectares	Optimal	Viable	Non-viable
WA	8,193,824	2,274,570	128,369,907
Qld	50,730,230	11,675,167	109,897,902
NT	15,020,282	3,185,570	115,234,148

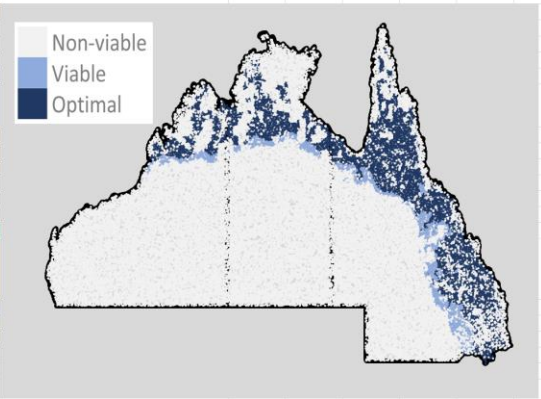


Figure 2. Example use of the interactive spreadsheet. A. Screenshot showing the default start where all grazing land is defined as Optimal and all other land uses Non-viable. The hectares in each viability class are automatically calculated in the yellow cells. B. Screenshot, where rainfall is selected as the sole determining variable and partitioned Non-viable (<550mm), Viable (550-600mm) and Optimal (>600mm) by placing 1 in that row of the Choice column. This interactively reclassifies all points on the map and recalculates total hectares in the yellow cells. By trialling different combinations of variable and partition, experts could select the distribution that fitted their views on where cultivated leucaena was viable.

Table 2. Example point classifications. Two experts classify the same point using different variables and partitions. Expert 1 uses four layers all of which he rates as Viable except for the Woody Cover which is Non-viable at that point. By comparison Expert 2 uses all seven layers, six of which are Optimal with Woody Cover Viable. In both cases the Woody cover viability determines overall viability (bottom row) since in both these cases Woody Cover has the lowest viability of all layers at that point.

Layer	Expert 1	Expert 2
Soil pH	Viable	Optimal
Available water capacity	Viable	Optimal
Annual rainfall	Viable	Optimal
Dry season length		Optimal
Soil order		Optimal
Slope		Optimal
Woody cover	Non-viable	Viable
Viability	**Non-viable**	**Viable**

Economic analysis

An economic analysis was conducted subsequent to the distribution mapping to estimate the marginal economic benefits of additional leucaena adoption (Redlands and Other) in potentially suitable parts of the study area for 40 years (2021-2060). Analyses were conducted for each of the 14 Australian Agriculture Survey regions (AGSURF 2020) in the study area.

Marginal benefit in each region were calculated as the amortised Net Present Value (NPV) of planting leucaena over its expected economic life adjusted for the opportunity cost of the extra capital required, and the opportunity cost of the land use foregone. Calculations of economic benefit ignores the RD&E costs of developing cv Redlands (or Other leucaena) and only identify the marginal benefit of planting new areas of leucaena at the paddock level. The benefit of existing plantings of leucaena were ignored and hectares adopted in each region were new leucaena plantings from 2022. Estimation of marginal benefits within regions is derived from multiple sources (Kenny and Drysdale 2019, Peck *et al.* 2015, Bowen and Chudleigh 2019, 2021a, 2021b, Bowen *et al.* 2015, 2021).

The key variables and their definitions used in the economic analyses are listed in Table 3. Calculations were run in a spreadsheet developed for the project and each analysis used a number of either estimated or assumed values for these variables as detailed in Appendix 4. These marginal benefits were calculated at discount rates of both 5% and 10%.

Table 3. Variables and their definitions for economic analysis. Values per region are shown in Appendix 4.

Variable	Definition
Type	Redlands or Other leucaena cultivar.
Net benefit	Amortised benefit of adoption per hectare.
Avail ha	Total hectares of Viable land in the region.
Peak	First year at maximum adoption.
Peak years	Number of years at maximum adoption.
Peak ha	Adoption hectares at *Peak*.
Decline years	Number of years of declining adoption following final peak year.
% Start	Percent of Viable land newly cultivated in 2021.
% Max	Maximum percent of viable cultivated 2021-2060.
% End	Percent of viable land newly cultivated in 2060.
Adoption	Percent of *Avail ha* where adoption occurs per year (2021 – Peak).
Dis-adoption	Percent of *Avail ha* where adoption ends per year (last year at *Peak ha* - 2060). Due to adoption of newer technologies.

Results

This section outlines the results of both the potential distribution mapping (3.1) and the economic analysis (3.2)

Potential distribution mapping

Table 4 shows the estimated potential areas for Redlands and Other leucaena-grass pasture systems in northern Australia. Queensland accounts for more than 90% of this estimated area and Redlands and Other leucaena types areas were differentiated on long term average rainfall (Figure 3).

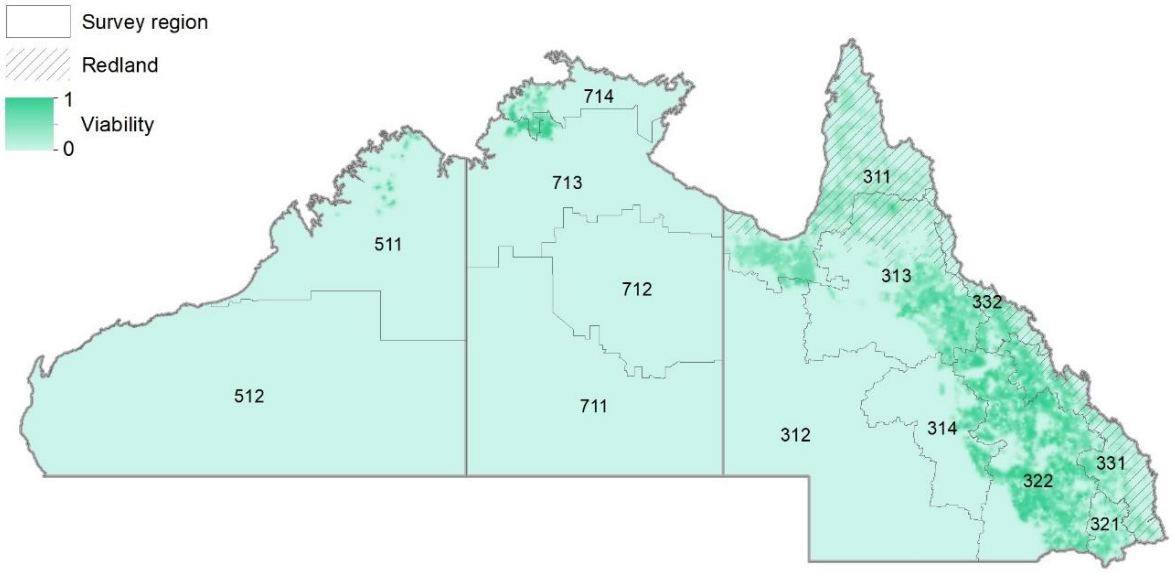
Table 4. Estimated viable hectares for cultivated leucaena–grass systems in the study area. Redlands and Other leucaena types areas were differentiated on long term average rainfall (Figure 3).

State	Type	Viable ha
NT	Other	1,306,944
Qld	Redland	4,679,553
Qld	Other	20,953,587
WA	Other	322,380
Total		**27,262,464**

Table 5 and Figure 3 detail the amount and distribution of potentially viable leucaena areas across northern Australia in each of the Australian Agriculture Survey regions. They reflect the far greater viable areas identified in Queensland as discussed above.

Table 5. Estimated viable hectares available for leucaena-grass pasture systems in Australian Agriculture Survey regions. Region numbers correspond to those listed in Figure 3. Any survey regions listed in Figure 3 and absent from this table had no viable leucaena areas.

State	Type	Region	Viable Ha
NT	Other	713	599,016
NT	Other	714	707,928
Qld	Other	311	1,691,506
Qld	Other	312	205,791
Qld	Other	313	3,667,612
Qld	Other	314	1,387,973
Qld	Other	321	752,030
Qld	Other	322	10,167,586
Qld	Other	331	2,396,976
Qld	Other	332	684,113
Qld	Redlands	311	1,887,005
Qld	Redlands	313	988,278
Qld	Redlands	321	58,590
Qld	Redlands	322	32,285
Qld	Redlands	331	1,144,993
Qld	Redlands	332	568,402
WA	Other	511	322,380



311: Cape York and the Queensland Gulf	332: North Queensland Coastal
312: West and South West	511: The Kimberly
313: Central North	512: Pilbara and the Central Pastoral
314: Charleville - Longreach	711: Alice Springs Districts
321: Eastern Darling Downs	712: Barkly Tablelands
322: Darling Downs and Central Highlands	713: Victoria River District - Katherine
331: South Queensland Coastal	714: Top End Darwin and the Gulf

Figure 3. Heat map of the potential distribution of viable leucaena cultivation areas in northern Australia. Mapped numbers indicate Australian Agriculture Survey Regions. Cross-hatched areas have average annual rainfall >800mm and viable areas within these areas were assigned to the Redlands leucaena cultivar. Please note, this map is for research purposes only, and should not be used to identify or select cultivation sites.

Economic analysis

Total NPV of leucaena adoption (Redlands and Other) for the entire study area over 40 years was estimated between $61M and $123M assuming 10% and 5% discount rate respectively. More than 90% of NPV will be accrued in Queensland reflecting the greater availability of viable land and projected adoption area in Queensland compared to other jurisdictions. Redlands added a NPV of $13M (10% NPV) to $26M (5% NPV) over the same period, adding more than 26% NPV to that generated by Other leucaena cultivation across the entire study area (Table 6).

Table 7 provides a more detailed picture of economic benefits per Australian Agriculture Survey region. In Queensland 75% of total NPV is expected to be generated in three regions; Darling Downs

and Central Highlands (322), North Central (313) and North Queensland Coastal (332), while in the Northern Territory, benefits are relatively evenly spread between Top End Darwin and the Gulf (714) and Victoria River District – Katherine (713). The benefits of cv Redlands cultivation are concentrated strongly (74%) in the coastal strip of Queensland south from Cairns (South Queensland Coastal (331) and North Queensland Coastal (332)).

Table 6. NPV generated by new (2021-2060) cv Redlands and Other leucaena cultivation across study area jurisdictions.

State	Type	NPV ($M)		Viable area	Used area
		5%	10%	(000,000 Ha)	(000 Ha)
Qld	Redlands	$26.05	$13.28	4.68	39.7
NT	Other	$3.57	$1.69	1.31	6.5
Qld	Other	$90.60	$44.62	20.95	136.2
WA	Other	$3.26	$1.43	0.32	3.2

Table 7. NPV generated by cv Redlands and Other leucaena adoption across the Australian Agriculture Survey regions within the study area. study area jurisdictions. Region numbers correspond to those listed in Figure 3. Any survey regions listed in Figure 3 and absent from this table had no viable leucaena areas.

State	Type	Region	NPV ($M)		Area available	Area used
			5%	10%	(000,000 ha)	(000 ha)
Qld	Redlands	311	$0.24	$0.14	1.89	0.9
Qld	Redlands	313	$4.39	$2.08	0.99	9.9
Qld	Redlands	321	$0.39	$0.18	0.06	0.6
Qld	Redlands	322	$1.84	$0.99	32.28	1.6
Qld	Redlands	331	$8.55	$3.62	1.15	22.9
Qld	Redlands	332	$10.64	$6.27	0.57	11.4
NT	Other	713	$1.99	$0.94	1.39	7.2
NT	Other	714	$1.57	$0.74	0.71	3.5
Qld	Other	311	$3.96	$2.33	1.69	16.9
Qld	Other	312	$0.23	$0.11	.21	1.0
Qld	Other	313	$16.80	$8.82	3.67	36.7
Qld	Other	314	$0.15	$0.07	1.39	0.7
Qld	Other	321	$0.25	$0.12	0.75	0.4
Qld	Other	322	$47.45	$21.83	10.17	50.8
Qld	Other	331	$8.94	$3.79	2.40	24.0
Qld	Other	332	$12.81	$7.55	0.68	13.7
WA	Other	511	$3.26	$1.43	0.32	3.2

Discussion

The results from mapping potential areas for leucaena cultivation suggest that a substantial part of northern Australia's grazing land could viably support leucaena-grass grazing systems. This finding aligns with previous analyses targeting various parts of the study region (Shelton and Dalzell 2007, Peck *et al.* 2011, Kenny and Drysdale 2019) which also indicated a large potential footprint for leucaena cultivation. This area is strongly skewed toward Queensland, however smaller but still significant areas were also identified in the Northern Territory and Western Australia.

A second key point from our spatial analysis is that in Queensland the introduction of the Redlands cultivar further extends potential cultivation areas for leucaena by 4.68M ha. These extensions are necessarily in higher rainfall areas (areas <800mm were assigned to Other leucaena varieties) where psyllids have historically limited the potential of other leucaena varieties. Redlands distribution was not modelled in the Northern Territory or Western Australia because psyllids don't appear to be a major factor for its viability in those jurisdictions.

The results of our mapping exercise are provided with two caveats. These caveats are explained below. This work does not consider the limitations or costs of leucaena cultivation in terms of the weed impacts in different regions and jurisdictions, though this is a risk in its adoption, and may result in costs being borne by land managers outside the cultivated properties. For example, Western Australia classifies leucaena as a very high environmental weed risk in the study area. This means it is not approved for use on pastoral leases (it can be cultivated on freehold land), which would limit its use on a large percentage of the landscape (Campbell *et al*. 2019). A sterile variety is in development (McMillan *et al*. 2019; Real *et al*. 2019; Revell *et al*. 2019) but not yet released. Our work does not take such circumstances into account. It should also be noted that this work does not explicitly account for the impact of local vegetation management laws in any jurisdiction. Leucaena – grass pastures should perform better in the absence of competition from other woody vegetation, and where substantial woody vegetation is present, clearing may precede cultivation if it is legal in the local jurisdiction. However, the complexity of vegetation management laws and their relationship with potential leucaena cultivation across three jurisdictions prevented us from taking vegetation management laws into account in this work.

Our estimates of potentially available cultivation areas would be lower if these two caveats were addressed, so the current estimates should be considered an upper limit on the estimated potential area. But even if these factors had significantly affected our estimates, it seems unlikely that their influence would be sufficient to affect the economic benefits discussed below since those analyses assumed very small areas of adoption relative to the potentially available area (discussed below).

The economics analysis suggests some benefits from adoption of both cv Redlands and Other leucaena – grass grazing systems ($61-123M across all jurisdictions), concurring with the general conclusions of other analyses (e.g. Shelton and Dalzell 2007, Bowen *et al.* 2018) that there are significant potential benefits to adoption of leucaena in appropriate circumstances. It should be noted too that these are only benefits from post 2020 plantings, and our results don't incorporate forward

benefits from existing crops. Total financial benefits were focussed in Queensland due almost entirely to the greater area potentially available. Other factors such as wider industry experience in cultivating leucaena in Queensland may also play a role but were not part of our analysis.

The economic analysis of cv Redlands adoption suggests it should add $13-$26M to Queensland beef businesses in the next 40 years, extending benefit in areas where the production benefits of leucaena have historically been difficult to achieve due to psyllid predation. Greatest benefits should accrue in high rainfall coastal strip south from Cairns where more area is available and where a higher density of grazing properties may make extension support more efficient. We did not project the adoption areas or economic benefits of cv Redlands in the Northern Territory and Western Australia given the lack of evidence that psyllid tolerance is needed at these locations. Should psyllids emerge as a problem in these jurisdictions, for example once sufficient leucaena is cultivated to sustain populations, then our projections would require adjustment. It should be noted though that new cv Redlands projections would simply be subtracted from the Other leucaena projections for those jurisdictions and total projections would not change.

The factor that played a particularly strong role in the results of the economic analysis is the estimated total area of adoption. In any region this was determined by assumed adoption and disadoption rates, and years at peak adoption, but never exceeded 2% of available area in any region. This projects an estimated 176,000 ha of additional leucaena adoption over the next 40 years in Queensland, and another 10,000 ha in the Northern Territory and Western Australia combined. While low, these numbers reflect historic adoption rates. Cultivars have been commercially available in Australia since 1962 (Lemke and Shotton 2021) and leucaena has been promoted in Queensland since at least the 1980s. Despite this, Buck *et al*. (2019) suggest only about 130,000 ha is planted across northern Australia.

Adoption can be driven by a number of factors, and while the current area of cultivated leucaena suggests that historical promotional efforts have so far generated somewhat limited adoption, other factors may have played a role. Leucaena is an expensive and problematic crop to establish and maintain (Shelton and Dalzell 2007, Buck *et al.* 2019, Shelton 2019), and given this, reluctance to adopt might be driven by a number of factors including reluctance to invest, lack of training or equipment, unwillingness to rest country during establishment or cultural inertia. These low rates of adoption and potential reasons for them are issues of active discussion within the beef industry (*e.g*. Buck *et al*. 2019). While the exact drivers and the fastest pathways to improved leucaena adoption are not clear, this seems a useful topic for further investigation.

Another clear takeaway from this work is that available area for leucaena cultivation in northern Australia isn't a limiting factor in future adoption of leucaena-grass pasture systems, including those incorporating the Redlands cultivar. Limited space has not been an historical problem and won't be the case in the foreseeable future. An abundance of viable land has two important implications, both of which should be seen as opportunities for industry. Firstly, there is scope for more carefully targeted extension and promotion to develop leucaena in higher viability areas to ensure better establishment and greater long-term productivity. Our mapping can provide broad guidance for these

programs, but more targeted mapping (e.g. Burgess 2010) and on-ground investigations are obviously justified at the enterprise scale to ensure best placement of this difficult to establish crop. A second point worth noting is that with ample space for leucaena cultivation there is also room to prevent weed leucaena spread by identifying and avoiding Leucaena establishment in higher risk areas. Diligent adherence to the industry code of practice (Christensen 2019) and, other guidelines for weed control, as well as adoption of a sterile cultivar (Revell *et al.* 2019) can all assist this goal and will enhance the stewardship credentials of adopters.

There are a number of avenues for future work in this area. As noted, our work doesn't provide sufficiently fine scaled analysis for use at farm scale to identify suitable areas for leucaena. It does however suggest broader areas that would be useful starting points for finer scaled planning involving agronomic and economic advisors. This planning could focus on optimal placement of leucaena at property scale, appropriate weed control, and more targeted analysis of potential financial outcomes.

Acknowledgements

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Appendices

List of Appendices

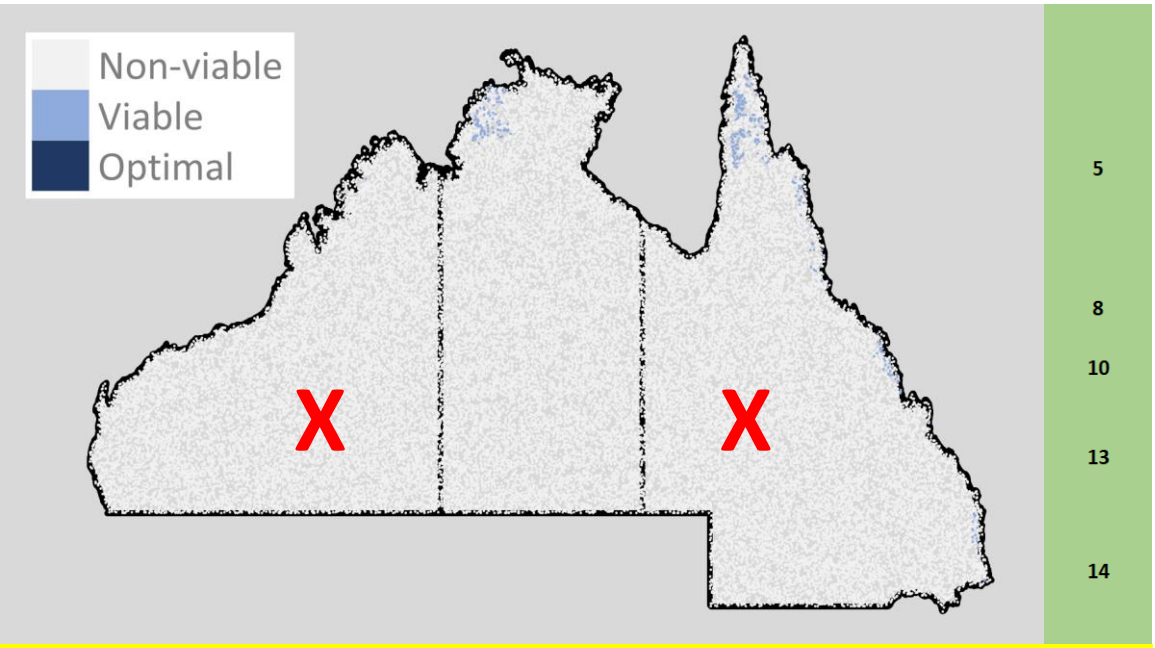
Appendix 1. Map layers used in potential leucaena distribution mapping.

Layer	Description	Source
Soil pH	Soil pH ($CaCl_2$) in top 60 cm of soil profile.	Soil and landscape grid of Australia. Grundy *et al.* (2015).
Available water capacity	Available water capacity in top 1m and 1.5m of soil profile.	Soil and landscape grid of Australia. Grundy *et al.* (2015).
Annual rainfall	Average annual rainfall.	BoM mean annual rainfall rasters (Product ID IDCJCM004)
Dry season length	Number of consecutive months with average monthly rainfall <20mm.	Derived from BoM mean monthly rainfall rasters (Product ID IDCJCM004)
Soil order	Soil order.	Digital atlas of Australian Soils. https://www.asris.csiro.au/themes/Atlas.html
Slope	Percent slope.	Grundy *et al.* (2015).
Woody cover	Persistent green derivative of fractional cover.	Gill *et al.* (2017).

Appendix 2. Expert panel mapping solutions.

Below are the potential distribution mapping solutions provided by each expert advisor. For each advisor non-relevant jurisdictions are indicated by a red cross and results for those jurisdictions were not included. Yellow cells indicate the total area of each viability class in the expert's jurisdiction. Green cell values indicate the variables/partitions used in the expert's solution, and these correspond to values in the *Choice* column of Table 1.

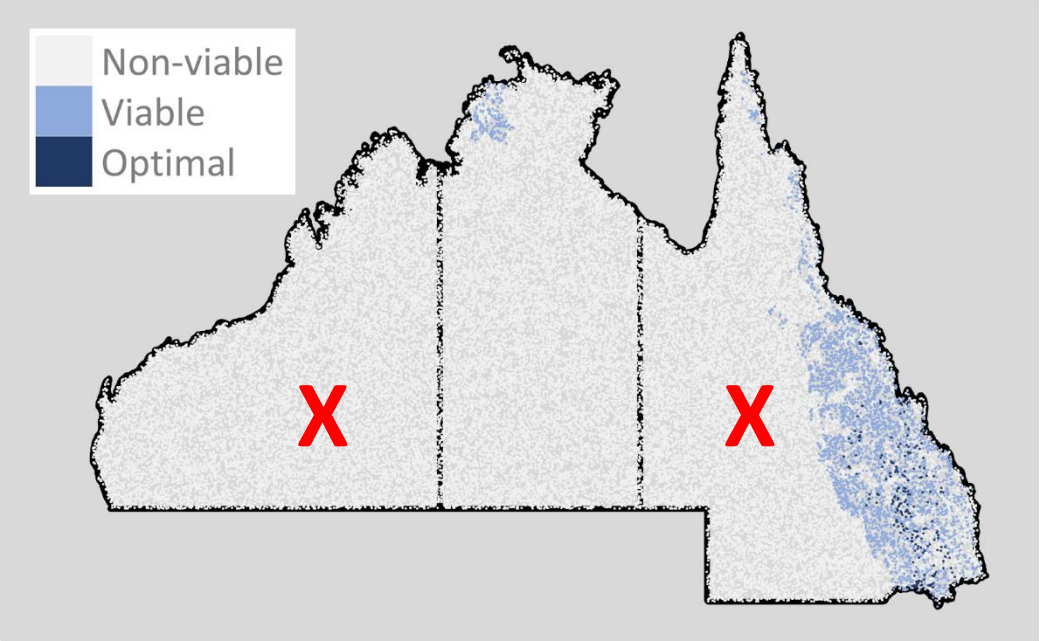
Expert: Arthur Cameron*. **Jurisdiction:** Northern Territory.



	Hectares	Optimal	Viable	Non-viable
	NT	0	925,721	132,514,279

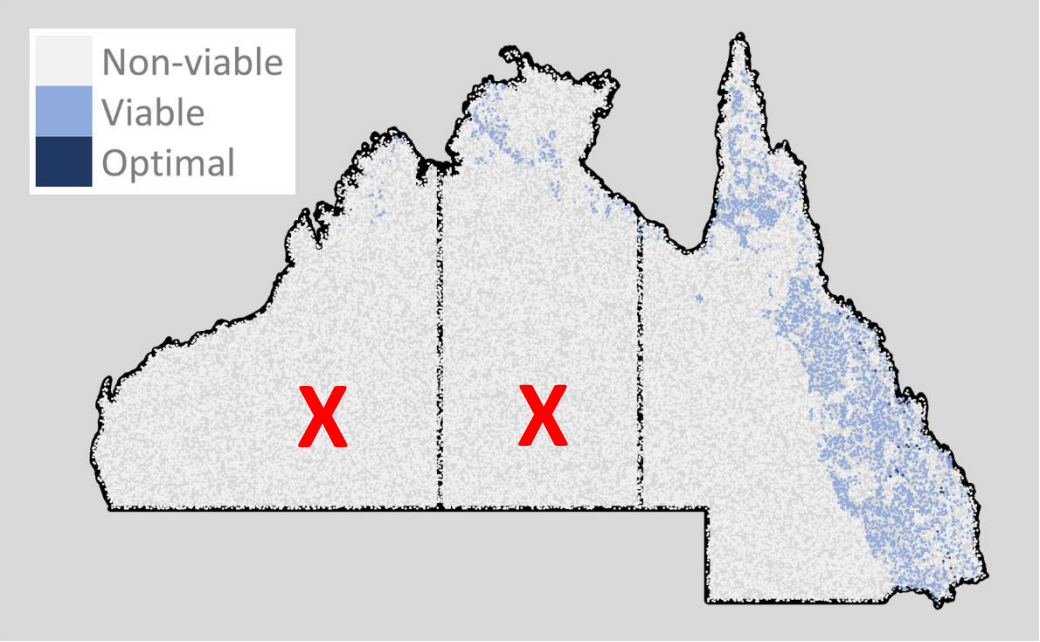
*This expert was not involved in initial development of partitions and requested an alternative annual rainfall partition (non-viable <1200mm, viable>1200mm) after development of the spreadsheet tool. The partition was included manually to meet the request, and all maps and data in the report incorporate this change.

Expert: Peter Shotton. **Jurisdiction:** Northern Territory.



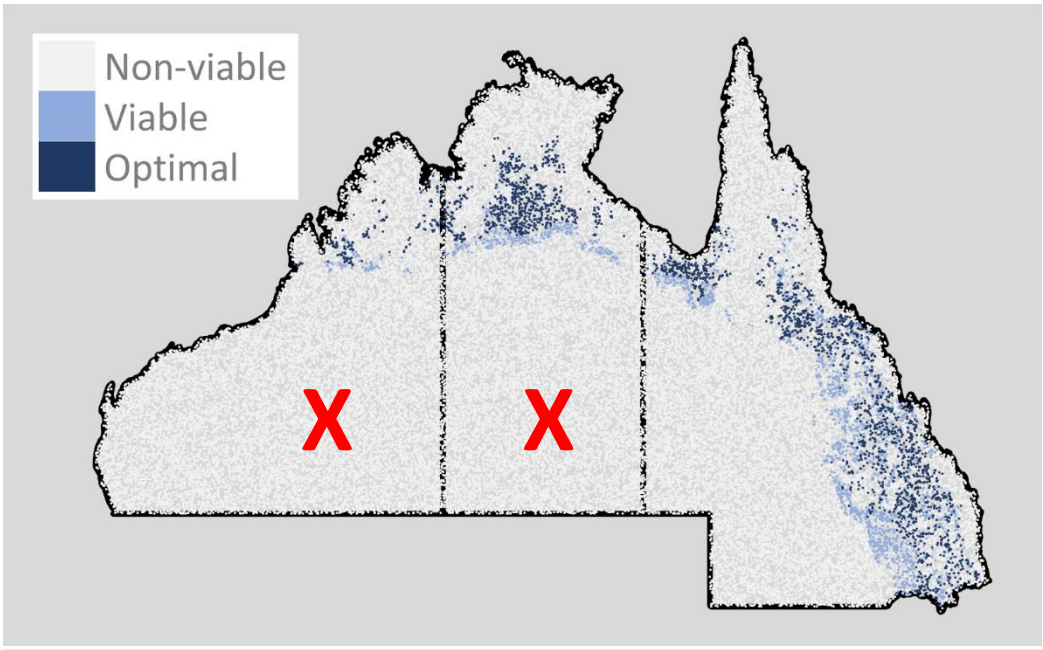
	Hectares	Optimal	Viable	Non-viable
	NT	0	1,688,080	131,751,920

Expert: Stuart Buck. **Jurisdiction:** Queensland.



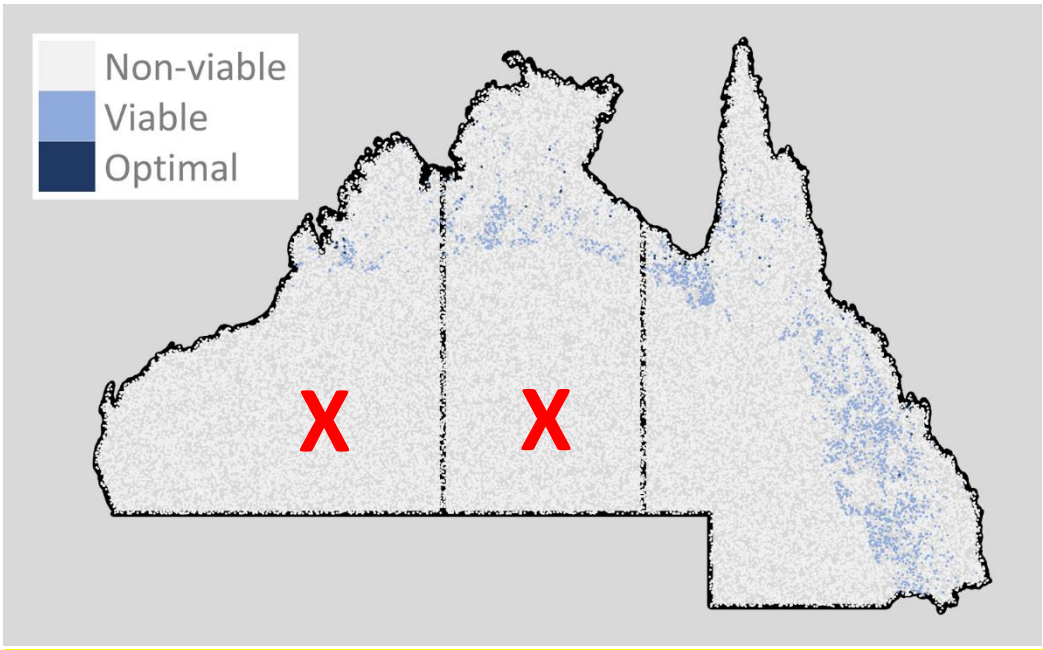
	Hectares	Optimal	Viable	Non-viable
	Qld	255,147	34,277,658	137,770,495

Expert: Scott Dalzell. **Jurisdiction:** Queensland.



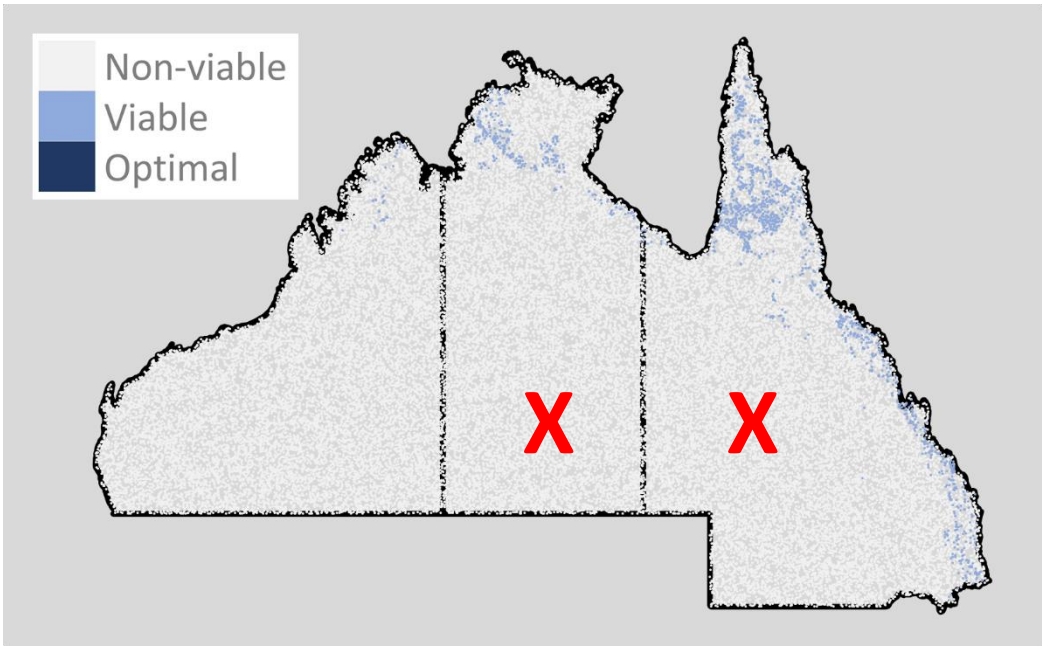
Hectares	Optimal	Viable	Non-viable
Qld	14,068,269	14,182,645	144,052,386

Expert: Joe Rolfe. **Jurisdiction:** Queensland.



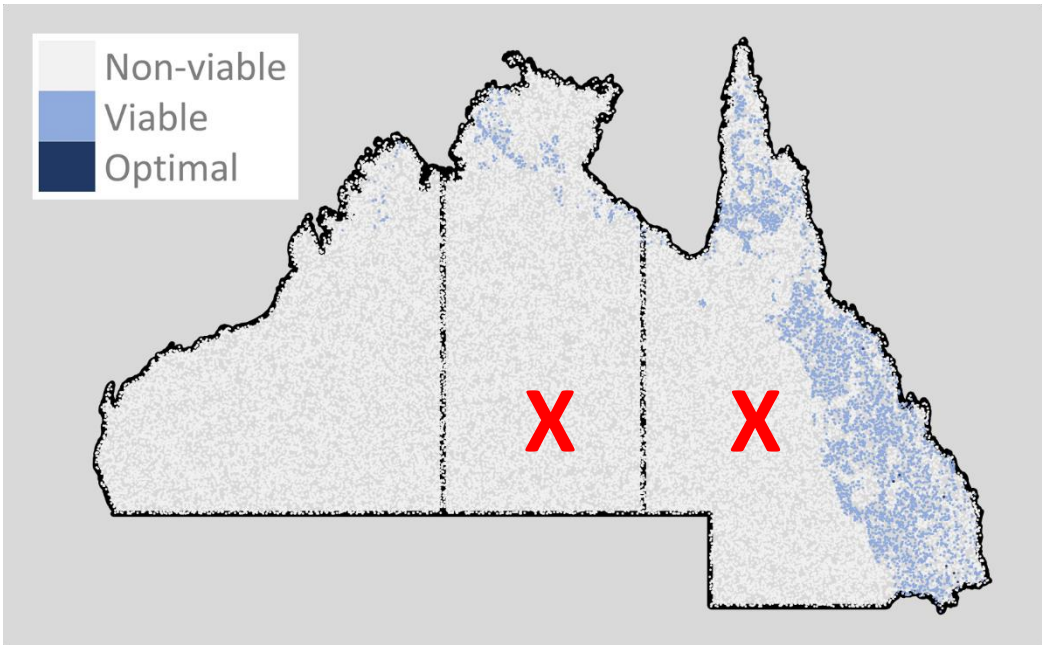
Hectares	Optimal	Viable	Non-viable
Qld	202,358	13,909,902	158,191,040

Expert: Geoff Moore. **Jurisdiction:** Western Australia.



	Hectares	Optimal	Viable	Non-viable
	WA	0	322,380	138,515,920

Expert: Clinton Revell. **Jurisdiction:** Western Australia.



	Hectares	Optimal	Viable	Non-viable
	WA	0	322,380	138,515,920

Appendix 3. Expert panel comments.

Below are the expert panel members' comments regarding their own solutions and the process used to map them. These were provided with the solutions in Appendix 2. All text is provided for transparency and does not necessarily represent the opinions of the report authors.

Expert: Arthur Cameron. **Jurisdiction:** Northern Territory.

Points which are important here in the NT are

- Rainfall, I would have another line for the NT, >1200mm and put a 1 in that column.
- High water table is important, not soil available water capacity. Leucaena does best where it can tap water during the dry season. Our soils run out of moisture during the dry season.
- It will grow in some of the drier areas, but it will not be a viable production system as it would not return the cost of development.
- In the NT, it is difficult to get permission to clear slopes over 2%.
- I do not see the relevance of the woody cover up here. If we are developing a high input pasture, the vegetation would be cleared in the process.

Expert: Peter Shotton. **Jurisdiction:** Northern Territory.

After trying several options on your calculator, the final was as attached. Although after re-visiting your draft from last year **"The potential distribution of Leucaena in northern Australia"** the NT appears closer to the NT mark in table 2 6,920 optimal, 80,575 viable. I think the water table depth (and water holding capacity) will have a big effect on the efficiency of leucaena as a high quality fodder species as it's the dry season leaf it produces for cattle production driven by moisture availability.
Leucaena growth in the Douglas-Daly (once established) thrives well in our 7 months (1240 mm) wet season and water table of around 15 meters keeps growing ok during the dry.

Expert: Stuart Buck. **Jurisdiction:** Queensland.

I'm still a bit surprised how little the optimal area is – across all states but particularly Qld. But maybe this is the reality and the current area planted, especially in CQ, is spread across both optimal and viable.

Expert: Scott Dalzell. **Jurisdiction:** Queensland.

Note: I did not think PAWC (due to coarseness/accuracy of the GIS data), dry season length (options too conservative), slope or woody cover were important (so didn't select an option for these characteristics).
On dry season length, I think you could modify categories – that dry seasons greater than 9 months are not viable. In the Gulf and Cape the wet season is only about 3 months long. But on deeper clay and loam soils leucaena does grow well on stored soil moisture carried over from the wet season. Leucaena is also used extensively in cut-and-carry feeding systems in Indonesia with similar monsoonal rainfall distribution.

Expert: Joe Rolfe. **Jurisdiction:** Queensland.

Here is my crack – key parameters for me are available water, rainfall and soil texture. I have also tossed in choices against slope and woody cover. As discussed, a cleared country variable could be included given changes to vegetation management laws are unlikely. Selecting and unselecting the slope variable doesn't change the Qld figures much.

Expert: Geoff Moore, Clinton Revell (combined). **Jurisdiction:** Western Australia

- The reality is that there is no commercial dryland leucaena in northern WA, only isolated plants which have naturalised - as a result our estimates are based on what we think will happen, rather than what we have observed.
- The assessment is on the basis of productive stands rather than plants just persisting
- Our assessment only applies to WA, we will let the NT and Queenslanders assess their states
- We were not expecting a large area suitable for growing dryland leucaena in northern WA; so 300K ha is in the ballpark we were expecting
- Establishment is an issue in WA - mature plants would persist and in most years be productive over the wet season in medium rainfall areas, however it is highly doubtful that leucaena rows (cf isolated plants) would establish and then persist through the first dry season without irrigation in those same areas.
- Question about the area of vertosols in the north Kimberley...? We were expecting to see an area on the map corresponding to the Ord irrigation area where dryland leucaena would grow (large area of vertosols). But no such area appears on the map.....?
- Q. Are you planning to super-impose land use (Pastoral lease, National Park/Conservation Reserve; Crown land) as an overlay within the GIS system to derive an area of leucaena on pastoral/freehold land?

Expert: Max Shelton. **Jurisdiction:** NA *

Thank you for sending me your spreadsheet with associated criteria for mapping lands suitable for growing leucaena. I can remember having a shot at a similar exercise more than a decade ago. We similarly based our maps on soils, fertility, and rainfall criteria.

While it seemed to us to be an excellent idea to estimate the area of land suitable for growing leucaena for policy purposes to inform future strategies, I have a number of caveats regarding this objective, purpose and use of the mapping. I went through your criteria and initially selected the first option for most categories and noted the result. These are my observations.

1. The overall outcome is a very large area of apparently suitable land, the majority of which will never be planted for many reasons other than suitability e.g. alternative uses, not a cattle production area, different goals and aspirations of the land owners, national park or amenity lands, other community uses etc. In this sense, the gross numbers will be of limited value to policy makers.
2. At a more detailed level, the map may also be of limited value due to scaling limitations. The scale and level of detail of the underlying soil maps that feed into the suitability map may be too broad to be useful at a property level. For instance, in coastal regions, and indeed in all undulating country, there can be a mix of soil types with pockets of creek flats surrounded by less fertile shallow soils with poorer moisture status.
3. I have just spoken with two graziers who are using the new cv Redlands to great advantage on coastal properties. In both case they are exploiting creek flats to great advantage.

   Regarding the specifics of your exercise, I formed a number of queries from the resulting map.

4. There was a concentration of viable planting environments in sub-humid zones (the agricultural zone) which, in the past, would not surprise me. However, with the availability of psyllid resistant cv. Redlands, much of the coastal land (the old spear grass zone) comes into play. I assume that it is excluded for reasons of unsuitable soil category which may not now be relevant. We have always known that water availability in marginal rainfall zones was important to successful leucaena establishment which meant that good soil depth and water holding capacity were important. But the coastal zones have higher and more reliable rainfall so water holding capacity is less important. In addition, new techniques are being used in coastal plantings such as planting on raised beds which have lessened the impact of water logging, poor fertility, and poor soil depth.
5. There are no criteria for soil fertility apart from the soil type. Low soil fertility is a huge impediment to successful establishment of productive leucaena. While low phosphorus and sulphur are major limitations in much of the brigalow, downs, and basalt country; in coastal country, there is a greater range of potential nutrient limitations including as P, K, S, Zn, Cu, Mo. I do realise that nutrient limitations can be overcome with fertilizer application, but so can water logging and poor soil depth by the use of raised beds. Lack of appropriate fertiliser use, both on older existing leucaena paintings as well as on newer plantings on poorer soils, may be one of the most important limitations to successful leucaena establishment and management going forward.
6. New planting techniques have even succeeded in planting leucaena on sloping land by the use of terraces with raised beds and banks which capture water flows.

   These questions are important as the release of cv. Redlands has opened up much new coastal land for leucaena panting where the existing grasslands are nutritionally very poor and there is now an

option for massive improvement of production and profitability. It would be counter-productive to produce maps indicating lack of suitability when the reverse is true provide new planting and management techniques are implemented.

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*NA – Not applicable as did not provide map solution

Appendix 4. Settings for economic analysis.

Below are the settings used in the economic analysis. Key variable definitions are provided in Table 3.

State	Type	Region	Net benefit	Avail ha	Peak	Peak years	Peak ha	Decline years	% Start	% Max	% End	Adoption	Dis-adoption	NPV 5%	NPV 10%
NT	Other	713	$75	599,016	2041	10	2,995	5	0%	0.50%	0%	0.025%	0.10%	$1,994,274	$944,466
NT	Other	714	$50	707,928	2041	10	3,540	5	0%	0.50%	0%	0.025%	0.10%	$1,571,246	$744,125
Qld	Other	311	$25	1,691,506	2031	10	16,915	5	0%	1.00%	0%	0.100%	0.20%	$3,959,122	$2,334,014
Qld	Other	312	$25	205,791	2041	10	1,029	5	0%	0.50%	0%	0.025%	0.10%	$228,377	$108,157
Qld	Other	313	$50	3,667,612	2036	10	36,676	5	0%	1.00%	0%	0.067%	0.20%	$16,800,953	$8,817,064
Qld	Other	314	$25	1,387,973	2041	10	694	5	0%	0.05%	0%	0.003%	0.01%	$154,030	$72,947
Qld	Other	321	$75	752,030	2041	10	376	5	0%	0.05%	0%	0.003%	0.01%	$250,370	$118,572
Qld	Other	322	$100	10,167,586	2041	10	50,838	10	0%	0.50%	0%	0.025%	0.05%	$47,449,006	$21,835,451
Qld	Other	331	$50	2,396,976	2051	5	23,970	5	0%	1.00%	0%	0.033%	0.20%	$8,945,717	$3,786,951
Qld	Other	332	$100	684,113	2031	10	13,682	5	0%	2.00%	0%	0.200%	0.40%	$12,809,824	$7,551,754
Qld	Redlands	311	$25	1,887,005	2031	10	944	10	0%	0.05%	0%	0.010%	0.01%	$238,332	$135,731
Qld	Redlands	313	$50	988,278	2041	10	9,883	5	0%	1.00%	0%	0.050%	0.20%	$4,386,966	$2,077,618
Qld	Redlands	321	$75	58,590	2041	10	586	5	0%	1.00%	0%	0.050%	0.20%	$390,121	$184,757
Qld	Redlands	322	$100	32,285	2031	15	1,614	10	0%	5.00%	0%	0.500%	0.50%	$1,844,623	$990,374
Qld	Redlands	331	$50	1,144,993	2051	5	22,900	5	0%	2.00%	0%	0.067%	0.40%	$8,546,421	$3,617,918
Qld	Redlands	332	$100	568,402	2031	10	11,368	5	0%	2.00%	0%	0.200%	0.40%	$10,643,168	$6,274,449
WA	Other	511	$100	322,380	2041	20	3,224	5	0%	1.00%	0%	0.050%	0.20%	$3,256,799	$1,431,179