final report

Project Code: B.CCH.1067

Prepared by: Steve Hughes
Minister for Agriculture and Fisheries,
South Australian Research and Development Institute

Date published: July 2012

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

Variability in fermentability and methane production in lucerne (Medicago sativa)

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

milestone report

MLA project code:	B.CCH.1067
MLA project title:	Variability in fermentability and methane production in lucerne (*Medicago sativa*)
Project leader:	Steve Hughes
MLA project manager/coordinator:	Sarah-Jane Savage/Julian Hill
Milestone number:	6

Milestone
Final Report

Abstract
An in vitro rumen batch culture study was completed to compare the effect of lucerne genotypes and the effect of physiological maturity on methane (CH_4) production.

Executive Summary

Methane is the dominant agricultural greenhouse gas in Australia, the majority of which arises from enteric fermentation in ruminant domestic livestock. Lucerne is the most widely grown and important forage legume in Australia. Lucerne hay production in Australia is estimated alone to be worth over $ 280m pa and supports dairy, beef, sheep and horse production, as well as a growing export market.

Lucerne is widely used as a benchmark species to other forages or diets for their potential to mitigate CH_4. However, there are only very limited data available as to the effect of lucerne genotypes on CH_4 production. Practical tools for graziers to reduce methane emissions from extensive livestock production systems are extremely limited and few practical and cost-effective options for significant and persistent abatement have been developed. Evaluation of the amount and distribution of genetic diversity among accessions can enhance the genetic exploitation of lucerne by plant breeders. Increased knowledge and understanding of the potential of lucerne to decrease ruminal methanogenesis will ultimately assist producers in their ability to reduce greenhouse gas omissions, improve animal production and importantly improve farm profit.

The primary objective of this project (Experiment 1) was to investigate the effect of lucerne genotypes on *in vitro* methane production, with the objectives of determining if differences existed between them and if so identify genotypes with a high and low methanogenic potential and secondly (Experiment 2) establish whether differences in the methanogenic potential of a subset of genotypes persisted throughout the physiological development of the plant.

Experiment 3 within the project investigated the variation in methanogenic potential within a single accession (cv Aurora).

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

Aligned with all 3 Experiments was the objective to determine if any differences in methanogenic potential are associated with plant chemical and morphological characters

The outcomes of this pilot project will provide important baseline knowledge on which lucerne genetic resources offer the greatest potential for reducing CH_4 omissions and improve animal production. This trait variation, coupled with the mechanisms of action and molecular marker technology are of key importance for its improvement and will provide valuable future opportunities for selective plant breeding for impact mitigation, improved animal production and importantly improved farm profit.

Results to date suggest there is significant genetic variation in the chemical composition of lucerne. The implications demonstrate the potential to achieve a significant reduction in energy loses from the livestock industry.

WIP

Project objectives

The primary objective of this project (Experiment 1) was to investigate the effect of lucerne genotypes on *in vitro* methane production, with the objectives of determining if differences existed between them and if so identify genotypes with a high and low methanogenic potential and secondly (Experiment 2) establish whether differences in the methanogenic potential of a subset of genotypes persisted throughout the physiological development of the plant.

Experiment 3 within the project investigated the variation in methanogenic potential within a single accession (cv Aurora).

Aligned with all 3 Experiments was the overriding objective to investigate if any differences in methanogenic potential are associated with plant chemical and morphological characters – ie biological function.

Methodology

Germplasm

Thirty six entries of *Medicago sativa* (lucerne) germplasm, including 31 of s*ubsp sativa* (8 Australian commercial cultivars, 7 experimental lines and 9 plant introductions), 3 *nothosubsp.varia* (1 commercial cultivar, 1 experimental line and 1 plant introduction), plus 2 plant introductions of *subsp. caerulea* were selected for use in this project. Table 1 lists the accessions used in this experiment, their subspecies, country of origin and their principal attribute for selection. In additional to Australian commercial cultivars, accessions were selected on the basis of available Genetic Resource Centre characterisation data (data not presented) for a range in "best bet" traits reported to influence digestibility and herbage quality.

Experimental Design

300 seeds of the lucerne accessions were scarified and sown into Petri dishes between the 10th and 12th of June 2011. After 3-5 days up to 200 viable seedlings were transferred into seedling trays and grown in a glasshouse. Establishment of 4 accessions (Vernal, SA 45667, SA 32115 and SA 35169) was repeated on the 27th June due to an insufficient number of viable seeds. Plots of each of the 36 accessions were space planted in the field by hand between the 22nd and 24th

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

August 2011 (Fig 2). A randomised complete block design with 3 replicates was followed. Plots consisted of 50 plants from each accession using a 10 x 5 layout with 20cm row and column spacing. The dimension of each plot was 1.8m by 0.8m and the distance between plots was 1.5m across the length and width directions. Plots were covered with wire cages to prevent predation from birds. Weed & insect control was preformed prior to planting. Soil test levels of P and K were at recommended levels for lucerne.

Sample preparation

A cleaning cut on each lucerne plot was carried out on the 20th November 2011, using a sickle bar mower. No measurements were taken and the objective was to normalise all plots to minimise any potential differences in seed health, establishment and recovery.

Experiment 1: The objective was to determine if there is genetic variation in lucerne genotypes at equal stages of development. Primary vegetative growth from all 3 reps of 35 entries (*n* = 105 samples) were sampled on Dec18th 2011, approx four weeks after the cleaning cut, when there was sufficient biomass and the confounding effect of physiological maturity was minimised. The central 15 plants of each plot were cut at 3cm height above the ground, representing a vegetative development stage.

Experiment 2 investigated the effect of physiological maturity for *in vitro* fermentative traits on a subset of seven representative cultivars. Following methodologies as described by Kalu and Fick (1981,1983), herbage regrowth from the same central 15 plants sampled in Experiment 1 were individually sampled at 3cm above the ground when each plant represented additional morphological stages of development (flowering *n*= 21 and fruiting *n*=21). ***No data from this experiment is included in this report.***

Herbage from the 15 plants per plot from Experiments 1 & 2 were pooled, freeze-dried, ground to pass 1mm screen, using a Cyclotec 1093 grinder (CYCLOTECH 1093 Sample Mill, Tecator, Hoganas, Sweden) and formed the basis of physical, fermentability and chemical analyses.

Experiment 3 investigated the variation for *in vitro* fermentative traits within a single population of lucerne (cv Aurora). Sampling methodologies mimicked Experiments 1 and 2 above, except the herbage of the 15 plants per plot was not pooled. Individual plants (were collected at each vegetative (*n* = 45 samples), flowering (*n* = 45 samples) and fruiting (*n* = 45 samples) stages of morphological development. Herbage samples from the individual plants were freeze dried and due to limited quantities were ground with a knife grinder, which resulted in the material particle sizes greater > than 1mm. Subsamples of the same grinded lucerne material that is used for all further analysis.

Only data from the vegetative stages are presented in this report.

Material from all 3 Experiments was stored at room temperature (ie 20-25^{o} C) in sealed containers and formed the basis of physical, fermentability and chemical analyses.

Table 1. List of *Medicago sativa* accessions grown at the SARDI Genetic Resource Centre field nursery and used for the variation in fermentabilty study**.**

Entry	Subspecies	Country of Origin	Principle Attribute
G6701	nothosubsp. varia	Australia	Experimental - GHT tolerant parent
K202-11	subsp. sativa	Australia	Experimental – GHT tolerant
K203-11	subsp. sativa	Australia	Experimental - Resistant to Blue green aphid
SA 6,751	subsp. caerulea	Russian Federation	Introduction -High acid detergent fiber of the leaf at maturity
SA 6,772	subsp. sativa	Algeria	Introduction -Susceptible to Spotted Alfalfa aphid (*Therioaphis maculata*).
SA 32,115	subsp. sativa	India	Introduction -Low by-pass protein
SA 35,148	subsp. sativa	Morocco	Introduction -Low neutral detergent fiber of leaf at maturity
SA 35,169	subsp. sativa	Afghanistan	Introduction -Resistant to Spotted Alfalfa aphid (*Therioaphis maculata*).
SA 35,176	subsp. sativa	Spain	Introduction -High neutral detergent fiber of leaf at maturity
SA 43,070	subsp. caerulea	Iran	Introduction -Small leaf size
SA 45,667	nothosubsp. varia	Canada	Introduction -High by-pass protein
SA 45,668	subsp. sativa	United States	Introduction -High crude protein in leaf
SA 45,670	subsp. sativa	Afghanistan	Introduction -High crude protein in stem
SA 45,672	subsp. sativa	Morocco	Introduction -Large leaf size
SA 45,673	subsp. sativa	Turkey	Introduction -High neutral detergent fiber of stem taken at maturity
SA 45,674	subsp. sativa	Turkey	Introduction -Low neutral detergent fiber of stem taken at maturity.
SA 45,675	subsp. sativa	Bolivia	Introduction -Low acid detergent fiber of leaf at maturity
SA 45,676	subsp. sativa	Poland	Introduction -High acid detergent fiber of stem at maturity
SA 45,677	subsp. sativa	Lebanon	Introduction -Low acid detergent fiber of stem at maturity
SA 45,678	subsp. sativa	Russian Federation	Introduction -High stem to leaf ratio
SA 45,679	subsp. sativa	Sweden	Introduction -Resistant to pea aphid (*Acrythosiphon pisum*).
SA 45,680	subsp. sativa	India	Introduction -High unifoliate internode length
Aurora	subsp. sativa	Australia	Commercial
DT2-11	nothosubsp. varia	Australia	Commercial - Drought tolerant
Genesis	subsp. sativa	Australia	Commercial
Lahontan	subsp. sativa	United States	Experimental - Low saponins
Pampa	subsp. sativa	Argentina	Experimental - Low crude protein in stem. Low unifloiate internode length.
SARDI 10s2	subsp. sativa	Australia	Commercial
SARDI 7s2	subsp. sativa	Australia	Commercial
SARDI Grazer	subsp. sativa	Australia	Commercial
Sceptre	subsp. sativa	Australia	Commercial
UQL 1	subsp. sativa	Australia	Commercial
Varsat	subsp. sativa	Argentina	Experimental - Low stem to leaf ratio
Vernal	subsp. sativa	United States	Experimental - High saponins
WL 925HQ	subsp. sativa	New Zealand	Commercial
Yonje	subsp. sativa	Iran	Experimental - Resistant to Bacterial wilt (*Corynebacterium insidiosum*).
36 Entries	**3 Subspecies**	**17 Countries**	

Location and soil type

The field site is located in the SARDI Genetic Resources field nursery, at the Waite Institute, Urrbrae, South Australia. The fine sandy loam at this site is a red-brown earth (Stace *et al*. 1968) of the non-sodic Urrbrae series (Litchfield 1951). The upper 0.10m contains 18% clay, increasing to 32% in the A2 horizon (Prescott 1931). Soil pH is 5.9 (CaCl2) and there is a negligible amount of calcium carbonate (Grace *et al*.

1995). The site has subsurface drip irrigation, with lines running 100 cm apart, 20 cm beneath each plot, and drip intervals of 50 cm. Weekly applications of 30 mm/ha will be applied as required.

Physical Measurements

The methods to be used for measuring the physical vegetative growth parameters are presented in Table 2 for whole-plot measurements and Table 3 for individual plant measurements of herbage quality parameters (Humphries & Hughes 2006).

Table 2. Vegetative growth and whole-plant measurements used in evaluation of lucerne germplasm at the SARDI Genetic Resource Centre.

Descriptor	Frequency of measurement	Description
Establishment	1 month after planting	Number of plants established in each plot is recorded
Forage production	3 growth stages	Herbage cut at 3 cm from ground level and fresh weight weighed. Sampled for dry matter rate and conversion.
Morphological development stage	Before each cut and then twice weekly for Expt 2&3	A numerical scale as described by Kalu and Fick (1981, 1983). *Advancing maturity is associated with decreased quality measures of crude protein and digestibility.*
Stem length	Before every cut	Length of longest stem on a plant that represents overall plot (cm). *Stem length is a better indication of growth rates than plant height as it has an equal application for plants with a prostrate habit.*
Plant height	Before every cut	Vertical height of tallest stem on a plant that represents overall plot (cm). *Plant height is traditionally used to calculate winter activity*
Recovery rate	2 weeks after each cut	Length of longest stem on a plant that represents overall plot (cm). *Recovery rate is important in farming systems where a quick or slow response to cutting is required. Hay producers prefer slow recovery rates so that hay can be removed before new growth emerges, whereas dairy producers prefer quick recovery rates due to very short grazing rotations.*
Habit	4 weeks after primary herbage cut	Visual estimation based on a 1 (very prostate) to 6 (very erect) scale.

Table 3. Measurement of herbage quality parameters used in the evaluation of lucerne germplasm at the SARDI Genetic Resource Centre

Descriptor	Frequency of measurement	Description
Stem thickness[A]	Before every cut	The stem diameter is measured (cm) using electronic callipers at the 4th node from the base of the plant of 15 representative stems
Stem to leaf ratio[A]	Before every cut	Dry weight (g) of stem *v.* leaf of 15 representative stems that are hand cut at 3 cm above ground level
Internode distance [A]	Before every cut	Measured (cm) between the 4th and 5th node from the base of the plant of 15 representative stems
Number of nodes[A]	Before every cut	Number of nodes counted on 15 representative stems
Leaflet area[A]	Before every cut	Length and width (cm) of central leaflet measured using electronic callipers. Measurement taken on the 4th leaflet from the top on 15 representative stems. Converted to leaflet area using Scion Image.
Recovery bud number	2 weeks after primary cut	The number of new stems and vegetative buds initiating from the crown after 2 weeks of growth. Average of 5 plants per plot

[A] Measurements performed on the same 15 stems.

In vitro fermentability test (IVFT)

The fermentability of plants was examined in an in vitro batch fermentation system. One day prior to the experiment, 0.1 g of plant material was weighed in Bellco tubes and transferred to an anaerobic chamber (Coy Vinyl Anaerobic Chamber; Coy Laboratory Products Inc., USA) maintained at 39 ◦C and supplied with 800 mL/L N2, 100 mL/L CO2 and 100 mL/L H2, to expel the oxygen from the tubes. Inside the chamber, H2 was maintained at 30 mL/L throughout the experiment and there was no detectable O2, as monitored by Coy Oxygen and Hydrogen Analyser (Coy Laboratory Products Inc., USA).

On each of three measurement days, rumen fluid was collected from three fistulated sheep grazing oaten hay and supplemented with lupins (100 g/head/day, supplemented twice/week). After collection, rumen liquor was separated from the solids, pooled, transferred into the anaerobic chamber, buffered to pH 7.2 (McDougall, 1948), and 10mL of this mixture was dispensed into prepared Bellco tubes. A negative control (i.e., buffered rumen fluid only; NC) and a positive control (i.e., buffered rumen fluid and 0.1 g of oaten chaff; PC) were included in the assay as standards to identify differences in rumen fluid between runs. Each sample and the controls were prepared in triplicate. Inside the chamber, once the tubes were filled, they were sealed with a rubber stopper, crimped and incubated for 24 h at 39°C, with constant shaking at 50 rpm. At the end of the incubation, tubes were placed in a water bath at 39°C, and gas pressure was measured using a pressure transducer (Greisinger Electronic GmbH, Regenstauf, Germany).

At the end of the incubation, and after measuring gas pressure, 5mL of headspace gas was transferred to an exetainer tube (Labco, UK) for subsequent analysis of methane concentration by gas chromatography (Varian 3600, Varian, Inc., Palo Alto, A, USA). The instrument was fitted with a 60m HP-1 capillary column using He as the carrier gas. The injector temperature was 190°C, the column was held at a constant temperature at 37°C during analysis, while the flame ionisation detector temperature was 200°C. The methanogenic potential of the plants was expressed as total methane produced (mL)/g dry matter (DM) of feed incubated (Soliva et al., 2008). Bellco tubes were then opened and 1 mL of the liquid phase of the sample was mixed with 200 µL of 1M NaOH for volatile fatty acids (VFA) analysis. They were then analysed by GC, using an Agilent 6890 Series GC (Agilent Technologies Inc., Santa Clara, USA) with HP 6890 injector, capillary column HP-FFAP, 30 m X 1.0 µm, flame ionization detector (FID) and HP Chemstation software. Carrier gas was hydrogen gas with 6.6 mL per min., with oven T = 2400C, injector T = 2600C and detector T = 2650C. Also 1 mL of the liquid phase of the sample was collected and acidified with 200 µL of 2M HCl for ammonia analysis. The ammonia in the fermentation fluid was determined by spectrometry with a Boehringer Mannheim Test kit 1112732 (R-Biopharm, Darmstadt, Germany) on a Cobas Mira S autoanalyser (F Hoffman-La Roche Ltd, Basal, Switzerland). Plant materials were extracted as suggested by Sweeney *et al*. (2001), Neto *et al*. (2005) and Monthana and Lindequist (2005) with minor modifications.

VFA, NH3 and A:P datasets are currently incomplete and will be analysed and reported at a later date.

The research complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Australian Government National Health and Medical Research Council, 2004), following approval by the CSIRO Centre for Environment and Life Sciences Animal Ethics Committee

In vivo nutritive value analyses

The concentrations of OM and total ash were determined according to the methods of Faichney and White (1983). Total nitrogen was determined by combustion using a Leco FP-428 N Analyser (Sweeney and Rexroad, 1987). Crude protein was estimated by multiplying total N by 6.25. Concentrations of neutral detergent fibre (NDF) and acid detergent fibre (ADF) were measured sequentially, according to operating instructions, using an Ankom 200/220 Fibre analyser (Ankom® Tech. Co., Fairport, NY, USA). Hemicellulose was calculated by NDF minus ADF.

In vitro dry matter digestibility (DMD) was estimated using the pepsin-cellulase digestion method based on Klein and Baker (1993). Samples were run in duplicate with a subset of 7 Australian Fodder Industry Association standards (consisting of lucerne and annual legumes) with known in vivo DMD (AFIA, 2007). Using these standards, the pepsin-cellulase DMD was linearly adjusted to predict in vivo DMD. The energy value of the sample (MJ/kg at the maintenance level of feeding) was estimated by the equation: M/D = (0.172*DMD) - 1.707 (Standing Committee on Agriculture, 1990)

Metabolic Profiling

Approximately 30 mg of the previously homogenized, ground lucerne were weighed in Eppendorf tubes (2 mL). 500 µL of 100% MeOH containing 13C6-Sorbitol (1.0 mg/mL, for TMS derivatisation) as a quantification standard was added to the sample and vortexed for 30 seconds. The mixture was extracted for 15 minutes at 70°C. 500 µL of MilliQ water was added to the pellet and vortex for 30 seconds and then centrifuged at 13000 rpm for a further 15 minutes. The supernatant was then transferred to Eppendorf tubes (2 mL). 25 µL aliquots for each sample were dried in vacuo. One derivatisation methodology was carried out.

TMS Derivatisation: BSTFA with 1% TMCS
N,*O-bis* (Trimethylsilyl)trifluoroacetamide with Trimethylchlorosilane

The dry residues were re-dissolved and derivatised for 120 minutes at 37°C (in 10 µL of 30 mg/mL methoxyamine hydrochloride in pyridine) followed by treatment with 20 µL of BSTFA and 2.0 µL of a retention time standard mixture (0.029% (v/v) *n*-dodecane, *n*-pentadecane, *n*-nonadecane, *n*-docosane, *n*-octacosane, *n*-dotriacontane, *n*-hexatriacontane dissolved in pyridine) for 30 minutes. Sample volumes of 1 µL were injected onto the GC column using a hot needle technique for splitless analyses. For split analyses a split ratio was 1:20 was utilized.

The GC-MS system used comprised of a Gerstel 2.5.2 autosampler, a 7890A Agilent gas chromatograph and a 5975C Agilent quadrupole mass spectrometer (Agilent, Santa Clara, USA). The mass spectrometer was tuned according to the manufacturer's recommendations using tris-(perfluorobutyl)-amine (CF43).

Gas chromatography was performed on a 30 m VF-5MS column with 0.2□□m film thickness with a 10 m Integra guard column (Varian, Inc, Victoria, Australia). The injection temperature was set at 250°C, the MS transfer line at 280°C, the ion source adjusted to 250°C and the quadrupole at 150ºC. Helium was used as the carrier gas at a flow rate of 0.8 mL/min.

The analysis of TMS samples was performed under the following temperature program; start at injection 70°C, a hold for 1 minute, followed by a 7°C min^{-1} oven temperature ramp to 325°C and a final 6 minute heating at 325°C (Split and Splitless GC-MSAnalyses).

Mass spectra were recorded at 2 scan s^{-1} with an *m/z* 50-600 scanning range. Both chromatograms and mass spectra were evaluated using the Chemstation program (Agilent, Santa Clara, USA). Mass spectra of eluting TMS compounds were identified using the public domain mass spectra library of Max-Planck-Institute for Plant Physiology, Golm, Germany (http://csbdb.mpimp-golm.mpg.de/csbdb/dbma/msri.html) and the *in-house* Metabolomics Australia mass spectral library. All matching mass spectra were additionally verified by determination of the retention time by analysis of authentic standard substances. Resulting relative response ratios normalized per gram extracted fresh weight for each analysed metabolite were prepared as described in Roessner et al., 2001.

Data were prepared as described in Roessner *et al*. (2001) and presented as *x*-fold compared to the reference (segregating null) which is set to 1 (SARDI 7 Series 2). If two observations are described in the text as significantly different, this means that their difference was determined to be statistically significant ($P < 0.05$) according to the *t*-test algorithm incorporated into Microsoft Excel (Microsoft Corp., Seattle, USA).

Full metabolic quantification on SARDI 7 Series 2 Samples (3 replicates) is currently being carried out.

Tannin Analysis

The colorimetric tannin assay used is described in Li et al. (1996). Duplicate samples were analysed for each plant sample.

Pectin Analysis

The pectin assay used is as described by Filisetty-Cozzi & Carpita (1991) with modifications for insoluble plant cell walls by Dr Filomena Pettolino (Prof. Tony Bacic laboratory, University of Melbourne). The method provides a colorimetric estimation of uronic acids such as pectin with reduced interference from neutral sugars that brown during the sample preparation (hydrolysis step).

Analysis in incomplete and is currently in progress.

Statistical Analysis

For ‘between accession’ data, analysis of variance was carried out to determine the significance of difference between the accessions, using mean laboratory data for each of the three field replicate samples. No data transformation was necessary after inspection of the residuals. Correlations were used to demonstrate the relationships between plant characteristics. Principal components analysis (based on a correlation matrix) was used to demonstrate the differences between accessions for nutritive factors. The correlation matrix was used as plant quality measurements are on different scales. Vector values were superimposed on the biplot to show the value of specific variates and the direction of increase for values of that variate. The larger the variate arrows, the more important the variate is in separating the plants. For ‘within accession data’, AVOVA was not possible as individual plants represent individual genotypes. Gas production and methane data are plotted against one another.

Metabolic Profiling

For each batch an average of the three replicates was obtained which was then used for the determination of ratios. Further a percentage standard error to the mean was calculated and students t-test used to estimate statistical significance (P value

smaller than 0.05). To reduce the false discovery rate a further calculation called Bonferroni correction was carried out where the set P value of 0.05 was divided by the number of variables resulting in a new, more stringent P value. Data were prepared as described in Roessner et al. (2001) and presented as x-fold compared to the reference (segregating null) which is set to 1 (SARDI 7 Series 2). If two observations are described in the text as significantly different, this means that their difference was determined to be statistically significant ($P < 0.05$) according to the t-test algorithm incorporated into Microsoft Excel (Microsoft Corp., Seattle, USA). The normalized detected metabolite was then further converted into relative response ratios per gram fresh weight (area of analyte divided by area of internal standard ($^{13}C_6$-Sorbitol for TMS) and fresh weight of sample.

Full metabolic quantification on SARDI 7 Series 2 Samples (3 replicates) is currently being carried out.

Results & Discussion

Between accession data

There was significant variation between lucerne accessions for DMD (hence predicted M/D), CP, ADF, NDF, hemicelluloses and ash and all plant physical traits recorded. Tannin data is not reported as all samples had less tannin than the no-tannin control used in the analysis (effectively zero tannin).

We found no significant differences between accessions for gas production or methane gas per unit of DM. Plant physical traits showed very good relationships with DMD as expected. Means for accessions are presented in Table 4 & 5.

A possible explanation why we aren't seeing a relationship between total gas and DMD is that one system is based on a variable rumen fluid sample and the other a tightly controlled enzymatic (no microbial influence) digestion. In future when we're dealing with highly fermentable samples we are probably going to have to be much tighter in our design with the crude system than we are when we have a diverse range of plants that we're screening and have repeat samples through the screening systems for tighter standardisation.

Table 4. Mean nutritive traits of the 35 lucerne accessions compared

	Methane (mL/g DM)		Gas (kPa)		DMD (%)		Est. M/D	CP (% DM)		Ash (%)		NDF (% DM)		ADF (% DM)	
Accession	Mean	s.d.	Mean	s.d.	Mean	s.d.		Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
K202-11	60.3	9.70	118	7.0	72.5	1.92	10.75	23	1.3	11.5	0.29	28	3.1	21.1	2.29
SA 6772	65.3	22.48	109	8.9	71.4	0.72	10.57	25	0.9	13.6	0.44	31	1.2	23.1	1.03
G6701	38.2	14.53	106	13.2	71.2	0.40	10.54	26	1.6	12.7	2.04	30	5.0	22.7	2.73
SA 45674	49.6	6.86	110	1.3	71.2	1.01	10.54	27	2.0	11.7	0.82	30	4.4	22.7	2.07
Vernal	49.6	12.82	112	8.4	71.2	1.10	10.53	24	1.1	11.8	1.76	27	2.0	21.3	1.04
SA 45667	50.6	11.23	112	10.3	71.1	3.23	10.52	26	3.1	13.6	1.93	29	7.3	22.0	5.13
SA 35169	49.3	17.41	100	8.9	71.0	1.94	10.51	22	2.9	10.7	1.20	30	2.3	23.4	1.92
Pampa	42.8	16.93	107	11.7	71.0	0.97	10.51	23	0.5	11.2	0.52	29	3.6	22.5	2.87
SA 45675	51.8	7.82	111	3.5	70.9	4.19	10.49	23	0.9	11.7	0.62	27	7.4	21.1	4.82
SA 45677	43.5	6.25	103	1.8	70.9	0.30	10.49	26	0.9	13.0	0.24	32	2.6	23.5	1.71
SA 45668	49.5	13.12	109	10.3	70.9	1.06	10.49	24	0.4	11.5	0.62	31	0.2	23.6	0.99
SA 45678	55.4	27.57	114	8.4	70.2	0.20	10.37	25	1.0	12.2	0.46	28	2.7	22.0	1.49

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

Genesis	45.9	19.05	110	12.0	70.2	1.31	10.36	21	1.0	11.0	0.31	31	2.3	24.0	2.69
SA 6751	40.3	23.09	106	10.0	70.1	2.26	10.36	27	0.9	12.6	0.87	34	2.0	25.1	0.98
YONJE	38.4	7.10	104	6.5	70.1	2.70	10.36	23	2.3	10.6	0.42	32	4.7	25.1	3.63
UQL-1	47.3	11.61	109	4.5	70.0	3.78	10.33	22	0.7	11.5	0.59	32	4.4	23.7	2.78
SA 35148	47.6	20.90	116	10.3	69.8	2.12	10.30	24	0.2	12.1	1.55	30	4.1	23.0	2.50
SA 45679	54.3	8.24	105	15.4	69.8	2.63	10.29	22	0.5	11.3	0.49	30	2.7	23.1	1.79
Varsat	35.4	5.57	96	10.5	69.4	1.70	10.22	23	1.1	11.0	0.22	33	0.7	25.7	0.49
Lahontan	46.5	11.69	106	5.5	69.3	1.51	10.21	21	0.8	11.5	1.15	30	3.5	24.1	1.54
SA 35176	52.5	13.57	98	18.1	69.2	0.75	10.19	21	0.7	11.0	0.17	33	2.5	25.6	1.89
SA 45676	58.3	1.86	110	8.9	69.2	1.48	10.19	22	2.2	10.9	0.55	32	1.0	24.5	0.84
SARDI 7 s. 2	46.2	21.46	111	10.5	69.1	1.43	10.17	20	1.6	11.3	0.71	33	2.4	25.2	2.70
K203-11	46.8	14.71	102	15.6	69.0	2.49	10.16	21	0.9	11.9	1.27	32	1.9	25.2	1.27
SARDI 10 s. 2	41.5	8.14	106	2.8	69.0	3.27	10.16	21	2.4	11.9	0.95	32	0.9	25.2	1.05
Sceptre	48.0	9.72	110	8.7	68.9	0.77	10.14	22	1.3	11.0	0.39	31	3.1	24.9	1.51
SA 45672	49.5	18.75	111	10.2	68.7	2.14	10.12	19	1.3	11.6	0.80	32	3.6	24.7	2.88
SA 45673	44.4	12.31	113	5.7	68.7	1.69	10.10	24	0.7	11.5	0.42	31	2.2	24.0	1.47
SA 45670	55.3	12.72	111	11.6	68.2	2.05	10.01	23	2.3	9.9	0.64	34	1.8	25.6	2.24
SARDI Grazer	41.9	20.13	105	16.8	68.0	0.59	9.98	22	1.3	11.5	0.46	32	2.5	24.5	0.40
DT2-11	47.4	10.81	98	16.0	67.7	2.94	9.93	22	1.0	12.3	2.14	33	3.8	26.2	2.61
SA 32115	44.8	9.30	107	12.3	67.4	1.39	9.88	17	0.7	12.3	0.78	36	3.9	28.1	2.80
SA 43070	48.5	23.13	112	5.3	66.9	3.39	9.79	24	1.6	13.1	3.39	36	5.5	28.0	3.64
WL 925HQ	66.1	8.19	118	4.1	66.5	2.24	9.74	19	0.3	11.7	1.26	35	2.3	27.3	0.74
SA 45680	45.4	7.38	99	12.7	64.2	1.31	9.34	18	0.9	10.6	0.65	40	1.5	31.0	1.12
Grand Mean	48.5		108		69.5		10.25	23		11.7		32		24.4	
F prob	0.662		0.497		<0.001		<0.001	<0.001		<0.001		<0.001		<0.001	
LSD (5%)	21.06		15.52		2.92		0.50	2.20		1.49		4.16		3.19	

Table 5. Mean Plant physical traits of lucerne accessions at vegetative growth stage

Entry	Herbage	Days to Flower	Recovery	Habit	Stem Length (mm)	Stem Thickness (mm)	Internode Distance (mm)	No. Nodes	Leaf Width (mm)	Leaf length (mm)	Leaf Stem ratio
Aurora	5.138	20	31.41	4.67	38.24	1.04	23.3	10.44	2.278	22.3	0.78
DT2-11	4.686	15	20.42	2.96	25.89	0.89	18.18	10.27	1.987	16.56	0.86
G6701	4.666	19	17.93	2.77	24.4	1.07	20.13	10.47	1.926	13.38	1.05
Genesis	4.843	17	31.82	4.51	40.27	1.12	22.77	10.51	2.149	21.28	0.8
K202-11	4.741	19	28.51	4.40	34.58	1.08	24.11	10.24	2.209	20.3	0.88
K203-11	4.864	20	32.27	4.84	39.8	1.15	22.56	10.58	2.213	22.4	0.74
Lahontan	4.504	20	28.11	4.38	35.2	1.12	24.48	10.31	2.204	21.6	0.75
Pampa	4.532	26	20.71	3.67	25.29	1.2	15.99	10.84	1.902	15.22	0.79
SA 32115	4.445	16	34.98	5.95	47.26	1.36	29.04	11.32	2.063	21.56	0.52
SA 35148	4.557	20	18.27	2.87	23.21	0.98	18.52	9.88	1.982	17.09	1.04
SA 35169	4.783	17	33.67	4.96	36.64	1.27	25.52	9.42	2.339	24.99	0.83
SA 35176	4.588	20	32	4.59	37.79	1.42	26.16	9.75	2.308	24.53	0.8
SA 43070	4.816	11	23.73	1.00	34.04	0.87	37.54	11.11	1.128	11.86	0.59
SA 45667	4.648	21	20.64	2.96	25.78	1.02	19.84	10.02	1.915	16.25	0.92
SA 45668	4.859	15	23.8	3.07	32.27	1.04	21.88	10.44	1.914	17.29	0.84
SA 45670	4.768	20	30.4	4.36	36.51	0.98	24.63	9.64	2.167	19.94	0.74
SA 45672	4.820	15	35.18	4.96	40.84	1.32	27.97	9.84	2.348	25.28	0.71
SA 45673	4.939	20	22.64	4.16	36.96	1.29	29.95	9.64	1.913	15.46	0.68
SA 45674	4.835	23	19.62	1.02	27.91	0.93	19.24	13.38	1.258	12.29	0.65

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

SA 45675	4.495	17	22.41	4.10	32.99	0.96	25.78	10.36	1.88	18.46	0.73
SA 45676	4.895	19	29.53	4.33	36.8	1.42	25.95	10.49	2.104	22.39	0.82
SA 45677	4.648	14	17.11	1.53	22.29	0.94	20.35	11.42	1.385	10.85	0.95
SA 45678	5.049	19	21.42	2.87	29.09	0.9	22.66	10.73	1.873	15.12	0.9
SA 45679	4.793	18	27.58	4.16	33.82	1.28	24.08	10.13	2.144	21.16	0.84
SA 45680	4.848	17	38.64	5.89	52.02	1.16	29.34	11.27	2.098	23.27	0.52
SA 6751	4.756	19	16.29	1.78	27.36	1.2	29.75	10.56	1.206	12.87	0.73
SA 6772	4.305	19	13.72	2.13	19.11	1.01	20.26	10.29	1.641	11.6	1.18
SARDI 10 s.2	4.686	20	31.82	4.96	37.89	1.12	25.01	9.91	2.301	22.42	0.94
SARDI 7 s.2	4.795	16	29.2	4.71	36.02	1.22	25.86	9.96	2.223	21.05	0.85
SARDI Grazer	4.769	17	29.42	4.29	34.16	1.2	21.94	9.91	2.231	21.68	0.9
Sceptre	4.968	20	31.27	4.62	41.46	1.21	28.24	10.37	2.118	21.95	0.78
UQL-1	4.725	20	29.42	4.71	37.96	1.24	22.32	10.44	2.094	19.7	0.77
Varsat	4.883	17	26.4	4.18	36.51	1.05	20.93	10.98	1.885	17.9	0.75
Vernal	4.925	20	23.42	3.93	31	1.23	25.12	9.92	1.944	18.28	0.85
WL 925HQ	4.771	19	33.2	5.40	39.7	1.18	24.5	10.38	2.369	23.72	0.77
YONJE	4.725	19	30.13	4.56	36	1.12	20.01	9.58	2.207	20.36	0.8
Mean	4.735	18	26.59	3.89	34.08	1.13	24.01	10.41	1.996	18.95	0.81
F prob	0.045	<0.001	<0.001	<0.001	<0.001	<0.003	<0.001	<0.001	<0.001	<0.001	<0.001
LSD (5%)		3.5	3.51	0.42	5.75	0.28	6.27	1.12	0.191	2.61	0.14

Accession K202-11 had the highest DMD (72.5%) was statistically similar to 18 other accessions. Accession SA 45680 had the lowest DMD (64.2%). Using the ruminant feeding model GrazFeed (Freer et al 1997), it is predicted that a pregnant Merino ewe (day 100 of gestation) fed K202-11 would eat 1.2 kg of DM per day and grow at a rate of 210 g/week. In contrast the same ewe eating SA 45680 would eat 0.97 kg of DM per day and lose 112 g/week. For mature, non reproducing sheep, the difference in weight gain would be 3-fold (125 g gain/week on the lowest quality lucerne and 440 g/week for the highest quality lucerne). Estimated ME values are presented in Table 4 and range from 9.34 to 10.75 MJ ME/kg DM.

DMD was not correlated to methane production or total gas production. It was positively correlated to CP and (as expected) negatively correlated to ADF and NDF (Table 6). Crude protein ranged from 18 to 23 %, and all would meet the estimated crude protein requirements of reproducing ewes and growing lambs.

Table 6. Correlations between the nutritive traits measured

Methane	-						
Total gas	0.58***	-					
DMD	0.17	0.12	-				
ADF	-0.32***	-0.34***	-0.82***	-			
NDF	-0.32***	-0.34***	-0.78***	0.96***	-		
Hemi	-0.24*	-0.24*	-0.48***	0.60***	0.81***	-	
CP	-0.02	0.17	0.55***	-0.52***	-0.42***	-0.11	-
Ash	-0.10	0.03	-0.04	0.07	0.15	0.27**	0.25*
	Methane	Total gas	DMD	ADF	NDF	Hemi	CP

Significance of differences: *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$

Table 7. Correlations between plant physical traits measured and DMD

CH4_mL_g_DM	1	-					
DMD	2	0.0525	-				
Days_to_Flower	3	0.0337	0.3387*	-			
Development	4	-0.0464	-0.0839	-0.2986	-		
Habit	5	0.0183	-0.3973*	0.0918	0.0258	-	
Herbage	6	-0.0828	-0.1717	-0.0734	0.4161*	0.1444	-
Internode_Dist	7	0.0608	-0.4989**	-0.4520**	0.2046	0.1753	0.2170
Leaf_Stem_ratio	8	0.1625	0.5790***	0.1477	-0.0596	-0.3109	-0.3159
Leaf_Width	9	0.1492	-0.2326	0.1037	0.0391	**0.8654*** **	0.1482
Leaf_length	10	0.1203	-0.3729*	0.0003	0.1998	**0.8968*** **	0.2903
No_Nodes	11	-0.0803	-0.0326	0.0215	-0.0556	-0.4213*	0.0695
Plant_Height	12	-0.0101	-0.4950**	-0.0166	0.0685	**0.9624*** **	0.2587
Recovery	13	0.0222	-0.5463***	-0.1235	0.1751	**0.8635*** **	0.3705*
Stem_Length	14	-0.0608	-0.6347***	-0.1630	0.1404	0.8033***	0.3945*
Stem_thickness	15	-0.1318	-0.0379	-0.1616	0.2897	0.0383	0.1706
		1	DMD	3	4	Herbage	6

Internode_Dist	7	-					
Leaf_Stem_ratio	8	-0.5924***	-				
Leaf_Width	9	-0.0101	0.0104	-			
Leaf_length	10	0.2146	-0.2760	**0.9212*** **	-		
No_Nodes	11	-0.0663	-0.3646*	-0.6013***	-0.4429**	-	
Plant_Height	12	0.2845	-0.4599**	0.7982***	**0.8905*** **	-0.2966	-
Recovery	13	0.3872*	-0.5246**	0.7805***	**0.9087*** **	-0.2129	**0.9219*** **
Stem_Length	14	0.5628***	-0.7118***	0.5745***	0.7672***	-0.0642	**0.9097*** **
Stem_thickness	15	0.2065	-0.3869	-0.0959	0.0380	0.1288	0.0847
		7	Ratio	L width	L length	11	Height

Recovery	13	-	
Stem_Length	14	0.9295***	-
Stem_thickness	15	0.1057	0.1870
		13	14

Number of observations: 35
Significance of differences: *P* < .05, *, 0.335; *P* < .01, **, 0.431; *P* < .001, ***, 0.535

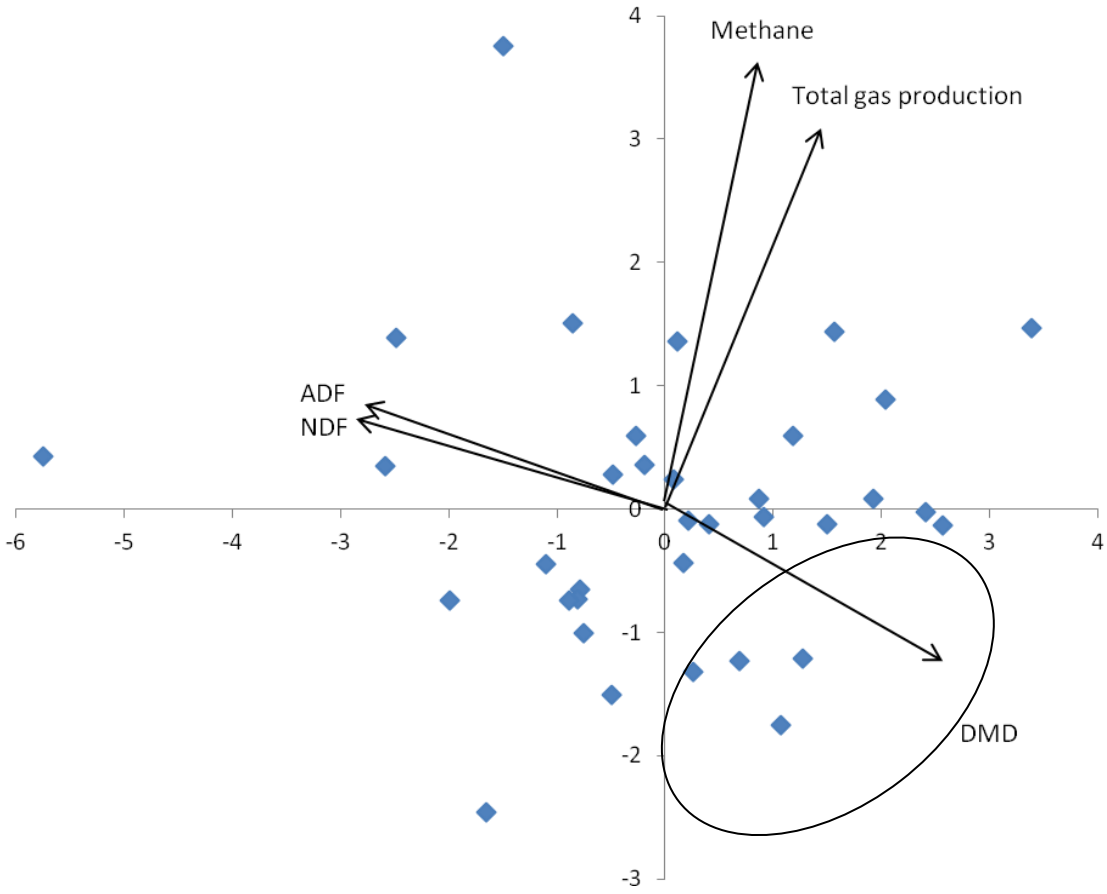

Figure 1. Principle Components Biplot showing variation between accessions across a range of traits. 88% of total variation is accounted for by the 5 variates included in the biplot.

Principal components analysis (based on a correlation matrix) demonstrates the differences between accessions for the digestibility factors (fibre, DMD, gas production and methane). Vector values have been superimposed on the biplot to show the value of specific variates and the direction of increase for values of that variate (Figure 1). The analysis also shows that DMD is negatively correlated to ADF and NDF. Methane and gas production are related and differ to DMD and fibre. Based on the data as it currently stands accessions with high DMD, low methane and low fibre would have the highest agricultural value (circled on the graph). These include G6701, SA45677, SA35169 and Pampa.

WIP

Within accession data

Figure 2 presents methane and total gas production of 45 individual plants originating from a single accession (Aurora). It is clear that plants differ in the methane emitted for each unit of gas production (as evidenced by a low R squared value for the correlation). Plants in the lower right quadrant of the graph appear to result in less methane per unit of gas produced and represent the most attractive option for plant improvement.

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

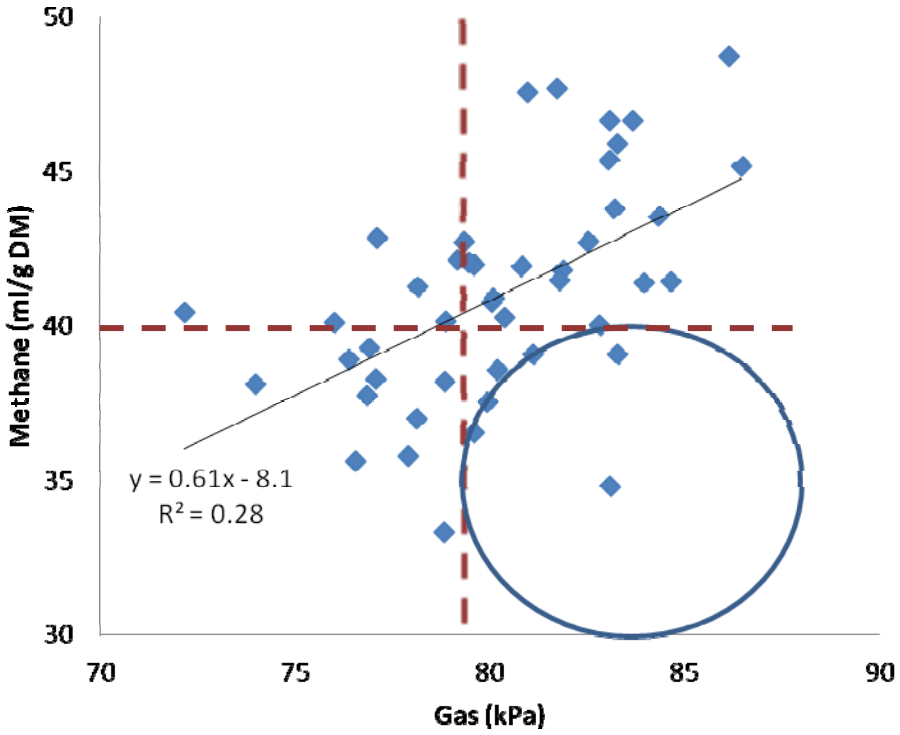

Figure 2. Methane and total gas production of 45 individual plants originating from a single accession (Aurora).

WIP

Metabolic Profiling

The resulting data comparing 35 batches (3 replicates) of lucerne have been provided as an excel sheet (Appendix 1). Values that are highlighted in green in Appendix 1 (with bold and italic formatting for good measure) have a *t*-Test value $P < 0.05$ / (number of metabolites) while those with cyan (and bold) highlight are between this *P* value and 0.05 (i.e. below 0.05, but not below the Bonferroni-corrected *P* value (Benjamini & Hochberg 1995).

WIP

Conclusions

These findings suggest there is significant genetic variation in the chemical composition of lucerne. The implications demonstrate the potential to achieve a significant reduction in energy loses from the livestock industry.

WIP

References

Barry TN, McNabb WC (1999) The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. *British Journal of Nutrition* **81**, 263-272.

Benjamini, Y & Hochberg, Y (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. Series B (Methodological). 57(1):289-300.

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

Cocks PS (2001) Ecology of herbaceous perennial legumes: a review of characteristics that may provide management options for the control of salinity and waterlogging in dryland cropping systems. Aust. J. Agric. Res., 2001, 52, 137–151

Durmic,Z., Hutton, P, Revell DK, .Emms J, Hughes S, Vercoe PE (2010). In Vitro Fermentative Traits of Australian Woody Perennial Plant Species that may be Considered as Potential Sources of Feed for Grazing Ruminants. Animal Feed Science.and Technology 160(3-4): 98-109

Grace PR, Oades JM, Keith H, Hancock TW (1995) Trends in wheat yields and soil organic carbon in the permanent Rotation Trial at the Waite Agricultural Research Institute, South Australia. Australian Journal of Experimental Agriculture 35, 857-864.

Humphries AW and Auricht GC (2001) Breeding lucerne for Australia's southern dryland cropping environments. Australian Journal of Agricultural Research 52 153 - 169

Humphries AW, Hughes SJ (2006) Preliminary Evaluation of Diverse Lucerne (Medicago Sativa Sspp.) Germplasm to Identify New Material for Livestock and Cropping Based Farming Systems In Australia. Australian Journal of Agricultural Research 57: 1297-1306.

Faichney GJ, White GA (1983) 'Methods for the analysis of feeds eaten by ruminants'. (CSIRO: Melbourne).

Freer, M., Moore, A.D., Donnelly, J.R., 1997. GRAZPLAN: decision support systems for Australian grazing enterprises. II. The animal biology model for feed intake, production and reproduction and the GrazFeed DSS. Agric. Systems 54, 77-126.

Kalu, B. A. and G. W. Fick (1981). "Quantifying Morphological Development of Alfalfa for Studies of Herbage Quality." Crop Science 21(2): 267-271.

Kalu, B. A. and G. W. Fick (1983). "Morphological Stage of Development as a Predictor Of Alfalfa Herbage Quality." Crop Science 23(6): 1167-1172.

Klein L, Baker SB (1993) Composition of the fractions of dry, mature subterranean clover digested *in vivo* and *in vitro*. In 'Proceedings of the XVII International Grasslands Congress'. pp. 593-594. (New Zealand Grasslands Association: Palmerston North)

Hall, M. B., B. A. Lewis, et al. (1997). "A simple method for estimation of neutral detergent-soluble fibre." Journal of the Science of Food and Agriculture 74(4): 441-449.

Li YG, Tanner G, and Larkin P (1996) The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. *Journal of Science in Food Agriculture* **70**, 89-101.

Litchfield WH (1951) 'Soil survey of the Waite Agricultural Research Institute, Glen Osmond, South Australia.' CSIRO, Division of soils divisional report. No.2/51, Adelaide.

McQuaker NR, Brown DF, Kluckner PD (1979) Digestion of environmental materials for analysis by Inductively Coupled Plasma-Atomic Emission Spectrometry. *Analytical Chemistry* **51**, 1082-1084.

Stace HCT, Hubble GD, Brewer R, Northcote KH, Sleeman JR, Mulcahy MJ, Hallsworth EG (1968) 'A handbook of Australian soils.' (Rellim Technical Publications: Adelaide, South Australia

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

Standing Committee on Agriculture (1990) 'Feeding Standards for Australian Livestock'. (CSIRO Publications: East Melbourne)

Sweeney RA, Rexroad PR (1987) Comparison of LECO FP-228 `N Determinator` with AOAC copper catalyst Kjeldahl method for crude protein. *Journal of the Association of Official Analytical Chemists* **70**, 1028-1032.

Van Soest, P. J., J. B. Robertson, et al. (1991). "Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition." Journal of Dairy Science 74(10): 3583-3597.

Van Soest P J 1994 Nutritional Ecology of the Ruminant (2nd edn). Cornell University Press, Ithaca, NY, USA.

Van Soest, P.J. 1995. What constitutes alfalfa quality: New considerations.p. 1–15. In Proc. 25th Natl. Alfalfa Symposium, Liverpool, NY. 27–28 Feb. 1995.

Zall DM, Fisher D, Garner MQ (1959) Photometric determination of chlorides in water. Analytical Chemistry 28, 1665-1668.

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

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