


# Increasing the proportion of *Leucaena leucocephala* in hay-fed beef steers reduces methane yield

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## ABSTRACT

**Context.** *Leucaena leucocephala* (leucaena) is a leguminous shrub adapted to higher rainfall (>600 mm) in frost-free areas of Australia. It can be a source of high-quality forage for cattle grazing tropical grass-based pastures that are seasonally deficient in the nitrogen content required for adequate levels of performance. Leucaena contains bioactive compounds that may reduce methanogenesis in the rumen, helping to achieve Australia's goal to make red meat production carbon neutral by 2030. **Aim.** A study was undertaken to evaluate the response in animal performance and methane production to increasing percentages of leucaena in a hay-based diet. **Methods.** Growing steers were fed diets containing 0%, 18%, 36% and 48% leucaena. Intake, liveweight gain, methane production and yield were measured in a cross-over trial with two modern cultivars of leucaena (Redlands and Wondergraze). Methane was measured in open-circuit respiration chambers. **Key results.** There were no effects of cultivar on most parameters. Increasing leucaena percentage in the diet increased dry matter intake, animal performance and methane production (g/day) but reduced methane yield (g/kg dry matter intake) according to the equation: methane yield =  $19.8 - 0.09 \times \text{leucaena percentage in the diet}$ . The inclusion of polyethylene glycol to nullify potential antimethanogenic activity of tannins restored methane yield by 67%, indicating that tannins were responsible for most of the observed reduction in methane yield. **Conclusion.** The results demonstrate that leucaena can improve animal performance and reduce methane yield in steers fed low-quality grasses. **Implications.** Leucaena can be included in diets of grazing cattle in areas agronomically suited to its production, as a means to reduce enteric methane emissions.

**Keywords:** Australia, cattle, greenhouse gas, intake, legume, methane, tannin, tropical.

## Introduction

In Australia, methane emissions from ruminant livestock are responsible for ~69% of total agricultural emissions of greenhouse gases (total was 69.7 Mt CO<sub>2</sub>-e in 2019) and 9.2% of the total national emissions (Australian Greenhouse Emissions Information System 2019). The Australian red meat industry has a goal of carbon neutrality by 2030 (Meat & Livestock Australia, 2021). *Leucaena leucocephala* (leucaena) is a leguminous shrub adapted to higher rainfall (>600 mm), frost-free areas of Australia. It can provide high-quality forage for cattle grazing tropical grass-based pastures (Tomkins *et al.* 2019). These C<sub>4</sub> grasses are typically deficient in nutrient content for adequate levels of performance, particularly in the dry season (Poppi and McLennan 2010). Shelton and Dalzell (2007) estimate that ~13.5 Mha of land is suitable for planting leucaena in the state of Queensland, which represents 9.2% of the grazing area in the state. Beutel *et al.* (2018) estimate that, in 2018, 123 500 ha of pastures contained leucaena. Leucaena has a deep and well-developed taproot, facilitating rapid growth and access to water and nutrient reserves in lower soil horizons than can be reached by grasses. As a result, leucaena is a productive, high-quality forage, typically having crude protein (CP) content of 15–25% (Shelton and Brewbaker 1998; Shelton and Dalzell 2007; Radrizzani *et al.* 2011). Bowen *et al.* (2016) compared six forage types in central Queensland and showed annual

liveweight (LW) gain to be 198 kg/ha for grass–leucaena pastures, which exceeded LW gain from other forages in the study, including oats (*Avena sativa*), sorghum (*Sorghum* spp.), lablab (*Lablab purpureus*) and mixtures of C<sub>4</sub> grass species–butterfly pea (*Clitoria ternatea*). Harrison *et al.* (2015) presented data comparing cattle grazing Rhodes grass pastures with and without leucaena and showed a 50% increase in LW gain over 14 months when leucaena was included in the pasture.

Antimethanogenic properties of leucaena have been demonstrated *in vivo* with cattle and sheep. Kennedy and Charmley (2012) reported an 18% methane abatement when leucaena was fed to cattle at 44% of the diet. Soltan *et al.* (2013) included leucaena at 35% in sheep diets and achieved a 14% reduction in methane emissions. Archimède *et al.* (2016) found that leucaena pellets included at 44% of a *Dichanthium* diet reduced methane production in sheep by 40%.

Tannins have been implicated in reducing methane emissions in ruminants (Jayanegara *et al.* 2012; Aboagye and Beauchemin 2019 (Aboagye and Beauchemin 2019)). However, the relationship between tannins and methane reduction is unclear and results can vary among studies (Aboagye and Beauchemin 2019). The concentration and molecular structure of tannins vary widely, with the major differentiation being between condensed tannins and hydrolysable tannins, the latter being more prevalent in tropical forages (Aboagye and Beauchemin 2019). Condensed tannins are often considered responsible for methane abatement in leucaena. Tannins could lower methane production by directly inhibiting methanogens and H<sub>2</sub>-producing microorganisms, and/or indirectly by reducing feed degradation in the rumen (Aboagye and Beauchemin 2019). The concentration of condensed tannins in leucaena is variable depending on a range of factors including subspecies and cultivar (Jones and Palmer 2002). Data for *L. leucocephala* show condensed tannin concentration ranging from 5 to 75 g/kg dry matter (DM) (Jones and Palmer 2002; Tan *et al.* 2011; Soltan *et al.* 2013; Archimède *et al.* 2016; Montoya-Flores *et al.* 2020).

The objectives of this study were to establish a relationship between the proportion of leucaena in the diet and methane production and feed intake, to determine whether there was a cultivar effect, and to demonstrate that inclusion of polyethylene glycol (PEG) in the diet reversed the suppressive effect of tannins on methane production.

## Materials and methods

The experiment was conducted at CSIRO's Lansdown Research Station (19°39'S, 146°50'E), 45 km south of Townsville, north-eastern Australia, from 18 March until 28 June 2019, with a further study when PEG was included

in the diet the following year from 13 June to 19 July 2020. Initially, it was planned that the inclusion of PEG to the diet would follow on from the main dose-response study. However, by mid-June the leucaena was exhibiting leaf loss and there was insufficient biomass available for the PEG study. The experimental protocols complied with the Australian code for the care and use of animals for scientific purposes (8th Edition 2013) and was approved by the CSIRO Queensland Animal Ethics Committee (AEC Numbers: 2019-02 and 2019-32).

Two cultivars of leucaena (Redlands and Wondergraze) were established at Lansdown Research Station in March–April 2017. The tropical legumes were sown into an existing grass pasture of 20 ha of mixed C<sub>4</sub> species dominated by Indian couch (*Bothriochloa pertusa*), Queensland bluegrass (*Dichanthium sericeum*), black speargrass (*Heteropogon contortus*) and sabi grass (*Urochloa mosambicensis*). Leucaena was established in February 2017 in twin rows spaced 12 m apart. Strips to be established with leucaena were sprayed with glyphosate (Roundup; Bayer, Leverkusen, Germany) and disced to provide a tilth suitable for seeding. The paddock was grazed for 3 weeks in April–May 2018 to remove excess biomass, and the leucaena rows were slashed to 30 cm above ground level in July 2018 to promote branching. Then, based on a soil report identifying a phosphorus deficiency (7 µg/g), the paddock was fertilised in September 2018 with 250 kg/ha of single superphosphate and 150 kg/ha of muriate of potash. Following these operations and favoured by an exceptional wet season (~1500 mm rainfall in January and February 2019), the leucaena plantation reached sufficient biomass to initiate the experiment in March 2019. Grasses in the sward were mowed in April 2019 to make the leucaena rows more accessible for harvesting.

Leucaena was hand harvested as stems cut at 1 m above ground level. Stems were transferred to a feed preparation area where green material (leaves and green stems <10 mm diameter) was removed by hand and shredded with a garden chopper (Greenfield herbage mulcher, Model GT03300; Cox Industries, Acadia Ridge, Qld, Australia). Thicker stems and mature pods were discarded. The leucaena was harvested and prepared 3 days per week (on Mondays, Wednesdays and Fridays) and stored in a cold room at <5°C until feeding.

## Experimental design and animals

Sixteen Droughtmaster (50% *Bos indicus*, 50% *Bos taurus*) steers with an initial LW of 428 ± 25 kg (mean ± s.e.) were used for the main experiment. After 4 months of grazing, they were housed in individual covered pens (3 m by 4 m) and allocated equally to four groups, based on methane emissions, recorded in a previous experiment.

Prior to introducing treatments, a 4-week period was used to familiarise the cattle with handling and with entry and exit

to the methane chambers, and for adaptation to Rhodes grass (*Chloris gayana*) hay. During this time, individual methane emissions were measured in open-circuit respiration chambers, creating a baseline methane production for each animal fed with 100% Rhodes grass at 90% of *ad libitum*. There followed a 2-week adaptation period, during which two leucaena cultivars were gradually introduced into the diet (cvv. Redlands and Wondergraze; eight steers per cultivar). On Day 7 of the adaptation period, each animal was dosed with an inoculum (100 mL) of a bacterial suspension of *Synergistes jonesii* as recommended by Business Queensland (Leucaena inoculum for cattle, Business Queensland: <https://www.business.qld.gov.au/industries/farms-fishing-forestry/agriculture/livestock/cattle/leucaena-inoculum-cattle>). After 14 days, leucaena inclusion levels of 0%, 18%, 36% and 48% were achieved, with steers remaining on these levels throughout four periods, each of 14 days. *Ad libitum* intake (10% refusals) was established within the first 7 days of each period; thereafter, intake was reduced to 90% of *ad libitum* to minimise variation in feed intake during methane measurements. After the first two periods, steers fed Wondergraze were switched to Redlands and *vice versa* with one extra week included in the design to adapt to the change in cultivars. Individual methane emissions were measured in open-circuit respiration chambers.

### Polyethylene glycol experiment

The PEG inclusion study utilised 12 Brangus steers (*B. indicus* × *B. taurus*) initially weighing  $325 \pm 9.8$  kg (mean ± s.e.), already adapted to the procedures and a hay–legume diet. Following a 7-day adaption to either cv. Redlands or cv. Wondergraze at 30% of the diet DM and inoculation with *S. jonesii* mixed culture, PEG4000 was dissolved in water and mixed with feed of eight (four on Redlands, four on Wondergraze) of the animals at 400 g PEG/day. Cattle were dosed once per day for the last 14 days of the 21-day period. Methane was measured in all 12 cattle on the last 2 days of the 14-day period.

## Diets and feeding

Steers had *ad libitum* access to water and diets. The basal diet consisted of Rhodes grass hay chopped (Roto-grind model 760; Burrows Enterprises, Greeley, CO, USA) to particle length of 5–10 cm to facilitate mixing with the fresh leucaena. A mineral–vitamin supplement was provided in the form of a lick block throughout the experiment (Olssons Trace element with copper and cobalt; Olssons, Yennora, NSW, Australia).

Just before feeding, the hay and fresh leucaena were prepared according to the four inclusion levels: 0%, 18%, 36% and 48%, on a DM basis. The diets were distributed between 09:00 and 10:00 hours. Feed intake was measured

throughout the experiment by calculating the difference between feed offered and feed removed 23 h later, adjusted for DM. Cattle were weighed weekly before feeding.

### Sampling and analysis

Samples of hay and leucaena offered and refusals were collected daily and bulked over 7 days when cattle were not in chambers, and over 3 days when cattle were in chambers, with the 3 days corresponding to the day before and the 2 days during which the steers were in chambers. Samples (10%) of the daily refusals were collected, and the samples pooled by animal.

Feed samples were dried in oven at 65°C, ground to 1 mm and packed in plastic bags before being sent to CSIRO Floreat Laboratory in Perth, Western Australia, for analysis. Ash, acid detergent fibre (ADF), neutral detergent fibre (NDF) and nitrogen (N) contents and DM digestibility (DMD) of feed samples were predicted by near-infrared spectroscopy (NIRS) using a scanning monochromator (Model 6500; NIRSystem, Silver Spring, MD, USA). The calibration database is large, about 1400 samples composed of tropical forages collected in Queensland. All samples (leucaena, hay, refusals) were predicted with the same prediction equations (Coates and Dixon 2011). The chemical composition of feed offered was computed from the proportional composition of the two forages according to their respective inclusion levels in the diet. The DM determinations of the bulked samples collected for the offered hay and leucaena, and refusals, were used to calculate DM intake (DMI).

Validation of NIR predictions was undertaken for N, the fibre fractions (ADF, NDF) and DMD by analysing 10% of samples by wet chemistry. Total N was determined using a CN 628 N analyser (LECO, St. Joseph, MI, USA). ADF and NDF contents were determined by the Ankom method (Ankom, Fairport, NY, USA) and DMD by the in-house pepsin cellulase method corrected for *in vivo* digestibility for cattle (Klein and Baker 1993). The  $R^2$  of correlations between wet chemistry and NIRS were found to be >0.93 for all analytes. Ash content of feed samples was measured by combustion at 550°C. CP content was calculated by multiplying N by 6.25, and hemicellulose was obtained by subtracting the ADF content from the NDF content.

Ruminal samples for pH, ammonia-N and volatile fatty acids (VFAs) were collected 3 h after feeding on Day 14 of each period, following the release of the steers from the methane chambers. The animals were restrained in a commercial cattle crush while the operator inserted an oral stomach tube. The pH was determined immediately, using a digital pH meter. Unstrained rumen fluid (4 mL) was preserved with 1 mL 20% metaphosphoric acid and stored frozen at –80°C prior to analysis for VFAs (Gagen *et al.* 2014) and ammonia-N (Chaney and Marbach 1962).

## Measurement of methane emissions

Four open-circuit respiration chambers were used to determine individual CH<sub>4</sub> and H<sub>2</sub> emissions. These chambers had an internal volume of 23.04 m<sup>3</sup> (4 m by 2.4 m by 2.4 m) and were constructed of a steel frame over which 4.10-mm clear polycarbonate was attached providing full visibility for each animal. A modified squeeze crush defined a confinement area in each chamber, and the floor was made of plastic grid allowing faeces and urine to flow into a container located below. Each chamber was equipped with a nose-activated drinking bowl and wheeled feed bin. Chambers were maintained at 2.0°C below ambient air temperature and under slight negative pressure (−10 Pa). Air was drawn from outside the building at a rate of 3000 L/min. Samples from the airflow coming into and out of the chambers were filtered, dehumidified, dried and refrigerated before analysis. The composition of air samples was determined by infrared for CH<sub>4</sub>, CO<sub>2</sub> and O<sub>2</sub> and by gas chromatography for H<sub>2</sub>. A full description of the components and functioning of these chambers is provided by [Martinez-Fernandez et al. \(2016\)](#).

For the baseline and each of the four 2-week periods, animals were held in individual pens from Day 1 to 11, transferred to open circuit respiration chambers on Day 12, and returned to individual pens on Day 14. Thus, methane emissions were collected over two consecutive days.

## Statistical analyses

All statistical analyses were performed using SAS software version 9.4 (SAS Institute, Cary, NC, USA). The individual steer was the experimental unit for DMI, gas (CH<sub>4</sub> and H<sub>2</sub>) emissions, LW gain, ruminal VFAs, ammonia-N and pH. The influence of the fixed factors (level of leucaena in the diet and cultivar) on gas emissions, DMI, and ruminal VFAs, ammonia-N and pH were analysed by using a mixed model procedure. The experiment used a split-plot design (cultivar and level), with polynomial analysis to assess the response (linear or quadratic) to leucaena inclusion level. With the exception of iso-valerate, cultivar had no effect on any variables measured, and excepting H<sub>2</sub> production, no interactions were observed between cultivar and level. With the exceptions noted above (Supplementary material Tables S1 and S2), the analysis was restricted to the main effect of level of leucaena inclusion.

## Results

### Cultivar effect

Both Wondergraze and Redlands are recently developed cultivars of leucaena selected for ease of establishment and yield. Redlands was commercially released in 2019 as a

psyllid-resistant cultivar. There were no cultivar effects for any of the parameters measured in this study apart from the exceptions noted above (Tables S1 and S2).

### Main experiment: diet quality, intake and performance

The Rhodes grass hay that constituted the basal diet was of poor quality as evidenced by high concentrations of fibre and low CP content. With DMD <50%, the hay quality was considered typical of dry season grasses in northern Australia ([Table 1](#)). The chemical composition of the freshly harvested leucaena (leaves plus stems <10 mm diameter) was similar in the two cultivars. As expected, the nutritive value of leucaena was superior to that of Rhodes grass, with CP content and DMD in excess of 14% and 60%, respectively.

Over the entire experimental period of 56 days, increasing the percentage of leucaena in the diet from 0% to 48% increased DMI according to a quadratic response ( $P < 0.001$ ) from ~13 to 17.5 g/kg bodyweight (BW) at 36% inclusion of leucaena ([Table 2](#)). There was no effect of cultivar on DM intake, but intake was apparently 5% lower when cattle were in chambers than pens. During confinement in chambers, treatment response in DMI and chemical components increased in a quadratic fashion ( $P < 0.001$ ), with greatest intake being achieved when leucaena was included in the diet at 36% ([Table 2](#)).

### Main experiment: rumen fermentation characteristics and gas production

With the exception of iso-valerate where the molar proportion was higher for Redlands than Wondergraze ( $P = 0.028$ ), cultivar had no effect on any of the rumen fermentation characteristics ([Table 3](#)). Total VFA concentration averaged 65 mg/dL across all inclusion levels of leucaena and

**Table 1.** Chemical composition of Rhodes grass (*Chloris gayana*) hay and leucaena (*Leucaena leucocephala*) cvv. Redlands and Wondergraze.

	Rhodes grass	Leucaena	
		Redlands	Wondergraze
<i>n</i>	12	18	18
DM (%)	86.48 ± 0.01	35.99 ± 0.03	34.72 ± 0.03
Ash (% of DM)	7.00 ± 0.29	7.50 ± 0.51	7.32 ± 0.73
CP (% of DM)	5.12 ± 0.39	15.36 ± 1.84	14.17 ± 1.52
DMD (%)	41.61 ± 1.47	61.34 ± 4.42	61.88 ± 3.14
NDF (% of DM)	73.09 ± 1.18	46.14 ± 2.07	45.05 ± 2.00
ADF (% of DM)	47.59 ± 1.56	32.50 ± 3.33	33.83 ± 2.85
Hemicellulose (% of DM)	25.50 ± 1.45	13.64 ± 3.03	11.22 ± 2.40

DM, dry matter; CP, crude protein; DMD, DM digestibility; NDF, neutral detergent fibre; ADF, acid detergent fibre.



**Table 2.** Nutrient intake and liveweight (LW) gain of steers throughout the 56 days of the experiment according to level of leucaena in the diet, and effect of location (pens vs chambers) on dry matter (DM) intake.

	Leucaena inclusion level in the diet (%)				s.e.	P-value	
	0	18	36	48		L	Q
DM intake: pens							
kg/day	5.25	6.96	7.93	7.46	0.18	<0.001	<0.001
g/kg LW	13.2	16.4	17.5	16.7	0.4	<0.001	<0.001
Nutrient intake: chambers							
DM (kg/day)	4.79	6.20	7.85	7.31	0.22	<0.001	<0.001
DM (g/kg LW)	12.2	14.9	17.3	16.4	0.4	<0.001	<0.001
CP (g/day)	257	474	721	745	19	<0.001	<0.001
NDF (kg/day)	3.48	4.13	4.87	4.33	0.15	<0.001	<0.001
ADF (kg/day)	2.25	2.70	3.26	2.94	0.10	<0.001	<0.001
LW gain (kg/day)	−0.52	0.12	0.45	0.41	0.12	<0.001	0.005

L, linear effect; Q, quadratic effect; CP, crude protein; DMD, DM digestibility; NDF, neutral detergent fibre; ADF, acid detergent fibre.

**Table 3.** Effect of leucaena inclusion in the diet on rumen volatile fatty acids (VFAs), ammonia-N ( $\text{NH}_3$ ) and pH.

	Leucaena inclusion level in the diet (%)				s.e.	P	
	0	18	36	48		L	Q
Total VFAs (mg/dL)	63.5	58.1	67.2	71.4	3.17	0.024	0.134
VFA (molar %)							
Acetate	75.6	74.7	75.8	74.1	0.965	0.461	0.660
Propionate	15.2	15.9	15.4	16.5	0.615	0.187	0.747
Iso-butyrate	0.480	0.464	0.449	0.538	0.0464	0.441	0.259
Butyrate	7.45	7.66	6.91	7.39	0.282	0.450	0.628
Iso-valerate <sup>A</sup>	0.592	0.496	0.516	0.563	0.3983	0.709	0.076
Valerate	0.584	0.716	0.713	0.748	0.0437	0.015	0.278
Caproate	0.151	0.113	0.128	0.113	0.0115	0.056	0.301
Acetate:propionate	5.14	4.82	4.96	4.57	0.186	0.065	0.854
$\text{NH}_3$ (mg/dL)	3.67	8.06	10.4	12.1	0.642	<0.001	0.042
pH	6.69	6.96	6.96	6.86	0.0468	0.021	<0.001

L, linear effect; Q, quadratic effect.

<sup>A</sup>Significant cultivar effect ( $P = 0.033$ ).

exhibited a positive linear response to leucaena inclusion ( $P = 0.024$ ). Proportions of VFAs were not greatly influenced by leucaena inclusion level, although there was a positive linear response for valerate ( $P = 0.015$ ). Rumen ammonia increased linearly ( $P < 0.001$ ) as leucaena inclusion level (and CP intake) increased. The pH of rumen liquor exhibited a quadratic response to leucaena inclusion, with higher values at the 18% and 36% inclusion rates ( $P < 0.001$ ).

As a consequence of increasing DMI, methane production increased as leucaena inclusion level increased (quadratic,

$P < 0.001$ ) (Table 4). However, when methane was expressed per unit DMI, methane yield decreased in a linear fashion ( $P < 0.001$ ).

Relative to results from the same animals during baseline measurements, methane yield was reduced by 15%, 20% and 22% at the 18%, 36% and 48% inclusion rates, respectively (Table 4). Production of  $\text{H}_2$  exhibited a positive linear response to leucaena inclusion level ( $P < 0.001$ ). However, there was a significant cultivar  $\times$  level interaction ( $P = 0.022$ ; Table S2) such that  $\text{H}_2$  levels were higher for Wondergraze at intermediate inclusion levels but not at

**Table 4.** Effect of leucaena inclusion in the diet on emissions of methane and hydrogen (H<sub>2</sub>) expressed per day and per unit dry matter intake (DMI).

	Leucaena inclusion level in the diet (%)				s.e.	P	
	0	18	36	48		L	Q
Methane							
g/day	94.4	111	126	114	2.72	<0.001	<0.001
g/kg DMI	19.9	18.1	16.2	15.8	0.385	<0.001	0.056
Baseline (g/kg DM)	19.5	21.7	20.2	20.2	0.099	–	–
Change from baseline (%)	+2.4	−14.9	−19.7	−21.6	2.09	<0.001	<0.001
Hydrogen							
mg/day <sup>A</sup>	237	190	326	464	46.6	<0.001	0.053
mg/kg DMI	49.8	31.3	41.9	64.0	7.71	0.127	0.011
Baseline (mg/kg DM)	41.6	31.1	33.8	31.5	0.011	–	–
Change from baseline (%)	+25.6	−81.5	+5.4	−53.7	40.85	0.413	0.558

L, linear effect; Q, quadratic effect.

<sup>A</sup>Significant cultivar × level interaction ( $P = 0.022$ ).

extreme inclusion levels. Yield of H<sub>2</sub> also increased with leucaena inclusion level, with the quadratic response being significant ( $P = 0.011$ ). Relationships are presented between leucaena inclusion level in the diet and DM intake (Fig. 1a), methane production (Fig. 1b) and methane production relative to baseline data (Fig. 1c). The relationship for methane yield was linear and can be defined as:

$$\text{Methane yield (g/kg DMI)} = 19.8 - 0.09 \\ \times \text{leucaena percentage in the diet}$$

### Polyethylene glycol experiment

The NIR analysis revealed higher nutritive value of both hay and the two leucaena cultivars in the PEG experiment than in the main trial. However, as with the main experiment, leucaena was of higher quality than Rhodes grass and differences between leucaena cultivars were small (Table 5). Including PEG in the diet did not influence DMI. However, methane production exhibited a numeric increase in response to PEG ( $P = 0.114$ ), which attained significance when expressed relative to DMI ( $P = 0.018$ ; Table 6). The pH was unaffected by PEG.

## Discussion

### Feed characteristics and animal performance

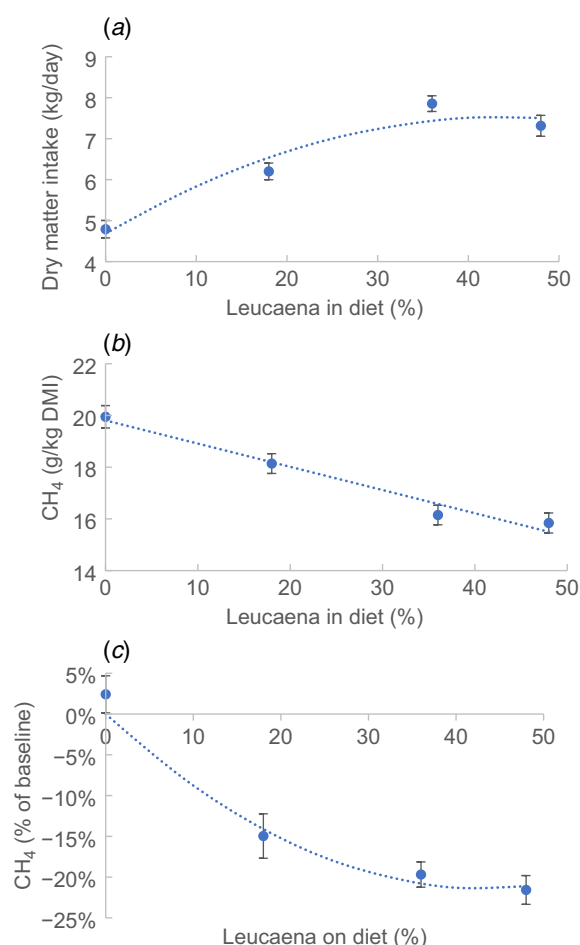
The composition and nutritive profile of the two leucaena cultivars was very similar but atypical of data recorded in the literature (Shelton and Dalzell 2007; Kennedy and Charmley 2012; Soltan *et al.* 2013; Archimède *et al.* 2016).

The low CP in the main experiment (~15%) was surprising and initially attributed to the inclusion of green stems (<10 mm diameter) in the diet. However, the same harvesting method was used the following season, and CP for both cultivars averaged 21%. The months following the 2019 wet season were dry, and the leucaena matured quickly, flowering and setting seed during the main experiment period from March to late June. By contrast, during 2020, out-of-season rainfall in May elicited fresh leucaena growth that coincided with the timing of the PEG experiment. The differences in CP content between years was attributed to the loss of leaf and high proportion of pods in the main study compared with the PEG study.

The Rhodes grass hay used as the basal diet in the main experiment was of poor quality, and this was reflected by the low DMI and LW gain exhibited in steers fed the 0% leucaena diet. Nevertheless, these nutritional characteristics were representative of dry season grass pastures in northern Australia. The response to increasing the proportion of leucaena in intake and growth was predictable considering the increase in N and metabolisable energy (ME) as the proportion of leucaena in the diet was increased. Similar results have been observed elsewhere (Hove *et al.* 2001; Soltan *et al.* 2013; Rira *et al.* 2015; Archimède *et al.* 2016; Molina *et al.* 2016). Exceptions to this intake response to leucaena are restricted to studies where the nutrient content of the diet has been held equivalent across treatments (Montoya-Flores *et al.* 2020).

### Effect of leucaena on methane production

Methane yields per kg DMI when feeding 100% grass hay during the baseline measurements (19.6 g/kg DMI) and for the 0% leucaena diet (19.9 g/kg DMI) were close to values described in the literature for non-methanogenic



**Fig. 1.** Changes in (a) dry matter intake, (b) methane (CH<sub>4</sub>) emission relative to dry matter intake, (c) and methane deviation from baseline according the level of leucaena present in the diet up to a maximum of 48%. Baseline is the gas emission by the same animals fed with 100% Rhodes grass (*Chloris Gayana*) hay. For (c), the curve was constrained to pass through the origin because the intercept was not significant.

**Table 5.** Chemical composition (mean  $\pm$  s.e.) of Rhodes grass (*Chloris gayana*) hay and leucaena cvv. Redlands and Wondergraze in the polyethylene glycol experiment.

	Rhodes grass	Leucaena	
		Redlands	Wondergraze
<i>n</i>	3	3	3
DM (%)	86.5 $\pm$ 0.2	34.6 $\pm$ 1.6	36.8 $\pm$ 1.2
Ash (% of DM)	8.12 $\pm$ 0.29	9.48 $\pm$ 0.25	8.95 $\pm$ 0.11
CP (% of DM)	9.27 $\pm$ 0.89	20.5 $\pm$ 0.04	21.6 $\pm$ 0.3
DMD (%)	51.9 $\pm$ 0.6	65.2 $\pm$ 0.7	66.9 $\pm$ 0.7
NDF (% of DM)	72.0 $\pm$ 0.4	34.8 $\pm$ 0.9	28.6 $\pm$ 1.4
ADF (% of DM)	41.6 $\pm$ 1.1	27.5 $\pm$ 0.9	27.2 $\pm$ 0.8
Hemicellulose (% of DM)	30.4 $\pm$ 0.8	7.3 $\pm$ 0.9	1.4 $\pm$ 0.8

DM, dry matter; CP, crude protein; DMD, DM digestibility; NDF, neutral detergent fibre; ADF, acid detergent fibre.

**Table 6.** Effect of polyethylene glycol (PEG) inclusion in the diet on dry matter intake (DMI) and methane emissions.

	No PEG	PEG	s.e.	P
DMI (kg/day)	6.71	7.07	0.45	0.526
Methane (g/day)	135	154	9	0.114
Methane (g/kg DMI)	21.3	23.0	0.5	0.018
pH	7.11	7.37	0.26	0.525

forages, using open-path respiration chambers (Kennedy and Charmley 2012; Hristov *et al.* 2013; Herd *et al.* 2014) but lower than those (23–27 g/kg DMI) for a tropical grass hay reported by Martinez-Fernandez *et al.* (2016, 2017). In a meta-analysis of published Australian data including dairy, temperate beef and tropical beef cattle fed predominantly forage diets, Charmley *et al.* (2016) obtained a single relationship of 20.7 g methane/kg DMI across an intake range of 3–28 kg/day.

Our results are characteristic of several studies where leucaena and other tropical legumes are added to low-quality grass diets that show methane production per head rising with increasing DMI, but methane yield per unit intake declining (Archimède *et al.* 2016; Molina *et al.* 2016; Suybeng *et al.* 2020). Typically, inclusion of the legume increases DMI as a consequence of increases in the N and ME concentration of the diet. The increase in methane production can be attributed to the increase in rumen available energy and retained N (Hove *et al.* 2001). When diet quality is equalised by formulating diets to be equivalent in N and ME, the intake response disappears and both methane production and yield decline (Montoya-Flores *et al.* 2020).

In the present study, the response to leucaena inclusion was measured in two ways: (i) by comparing the dose response relative to the control (0% leucaena in the diet) within each experimental period; and (ii) by measuring the response of individual animals relative to their baseline measurements taken at the start of the experiment. Although the results are broadly similar, the second approach indicated a curvilinear response. Beyond 36% leucaena inclusion, there was no further response in methane yield. These data suggest that an inclusion level of ~35% leucaena in the diet would provide the maximum benefit in methane avoidance. This is important because, under typical grazing conditions, leucaena may contribute only up to 40% of the dietary DM intake (Dalzell *et al.* 2012; Cardona *et al.* 2015; Bowen *et al.* 2016). Archimède *et al.* (2016) observed a 40% reduction in methane yield when sheep were fed leucaena leaf pellets at 44% of dietary DM, whereas Molina *et al.* (2016) observed a 17% reduction in methane yield when leucaena was included at 26% of the dietary DM of growing heifers. Montoya-Flores *et al.* (2020) observed a 14% reduction in methane yield for a similar level of leucaena inclusion. The results in the present study are

consistent with those of [Molina \*et al.\* \(2016\)](#) and [Montoya-Flores \*et al.\* \(2020\)](#), but the response found by [Archimède \*et al.\* \(2016\)](#) was nearly two-fold. This could reveal a difference between cattle and sheep, or could be related to the levels of bioactive compounds in the leucaena. If data from our experiment and from [Archimède \*et al.\* \(2016\)](#), [Molina \*et al.\* \(2016\)](#) and [Montoya-Flores \*et al.\* \(2020\)](#) are standardised to consider methane reduction at a theoretical 35% legume inclusion rate, the reductions in methane yield are 16%, 21%, 28% and 14% for the four datasets, respectively. This range in methane abatement gives some indication of the potential for leucaena to reduce methane emissions across a range of growing conditions and animal species.

Inhibition of methane, particularly when extreme, can lead to increases in hydrogen losses ([Martinez-Fernandez \*et al.\* \(2016\)](#)). The marked increase in H<sub>2</sub> production and yield for the 48% leucaena inclusion diet would suggest that the rumen had reached its potential to redirect hydrogen to alternative sinks such as propionate ([Martinez-Fernandez \*et al.\* \(2017\)](#)). Excess H<sub>2</sub> in the rumen could have been a factor in the quadratic response in performance and DMI because no benefits were observed in either variable for the 48% leucaena inclusion in the diet.

### Possible causes for methane reduction

Leucaena contains a number of plant compounds frequently cited as the bioactive agents in reducing methane emissions in ruminants ([Soltan \*et al.\* \(2013\)](#); [Aboagye \*et al.\* \(2019\)](#)). Foremost among these are tannins, a group of polyphenolic compounds broadly classified according to molecular structure and weight as condensed or hydrolysable tannins ([Huang \*et al.\* \(2011\)](#); [Aboagye and Beauchemin \(2019\)](#)). Tannin content can vary widely in leucaena according to the species ([Jones and Palmer \(2002\)](#)), and edaphic and climatic influences, management and growth stage ([Jayanegara \*et al.\* \(2012\)](#); [Aboagye \*et al.\* \(2019\)](#)). The relative concentrations of condensed and hydrolysable tannins may also vary, and because bioactivity is related to the type and molecular weight of tannins, this can influence the relationship between tannin concentration and methane production in the rumen ([Aboagye and Beauchemin \(2019\)](#)). For example, [Soltan \*et al.\* \(2013\)](#) concluded that the presence of condensed tannins (measured as leucocyanidin equivalents) in leucaena had no effect on methane yield. By contrast, [Archimède \*et al.\* \(2016\)](#) demonstrated a relationship between condensed tannin concentration (using quebracho standard) and methane reduction, with higher tannin concentration resulting in a greater reduction in methane yield. Quantification of tannins and their bioactivity is difficult ([Makkar \(2003\)](#)) and a biological measure of bioactivity using PEG can be used as a proxy by nullifying tannin bioactivity ([Soltan \*et al.\* \(2013\)](#)). This approach was chosen for the present study.

Blocking the action of tannins through the addition of PEG to the diet increased methane yield by 8% when leucaena was fed at 30% of dietary DM. This increase was smaller than expected considering the 13% reduction interpolated from the dose-response study and suggests that tannins may have accounted for approximately two-thirds of the methane reduction, but other mechanisms were also at play. However, the possibility that the PEG level was insufficient to nullify the entire tannin effect cannot be ruled out. In that case, the actual tannin effect may have been higher than observed. Tannins are thought to affect methane production through two main processes: a direct antimicrobial effect on methanogens and protozoa ([Tavendale \*et al.\* \(2005\)](#); [Animut \*et al.\* \(2008\)](#)), and an indirect effect on carbohydrate fermentation favouring reduced acetate and increased propionate through an effect on fibrolytic bacteria ([Rira \*et al.\* \(2015\)](#)). The effect of tannins on protein solubility in the rumen may further influence rumen fermentation by altering the balance between soluble carbohydrate and rumen-degradable protein for microbial protein synthesis ([McSweeney \*et al.\* \(2001\)](#)).

From the present study, it was concluded that approximately two-thirds of the methane reduction response could be attributed to tannins provided they were completely neutralised by PEG. The remaining one-third of the response could be related to changes in rumen fermentation in response to adding a high-quality legume. It is widely documented that methane yield is influenced by structural carbohydrate concentration in the diet. [van Lingen \*et al.\* \(2019\)](#) conducted a meta-analysis of 852 high-forage diets, and although DMI was the major determinant of methane production, NDF was the second most important. If the van Lingen equation ( $\text{DMI} = 23.8 + 13.5 \times \text{DMI} + 0.855 \times \text{NDF\%}$ ) is applied to the diets in our study, there was a 5% reduction in methane yield attributed to difference in NDF concentration between 0% and 35% inclusion of leucaena. This accounts for the 33% of methane mitigation that could not be attributed to tannins.

The influence of NDF on methane production in the rumen has been attributed to both physical and biochemical effects on methanogenesis. Reducing NDF in the diet increases rate of passage and reduces retention time ([Perry \*et al.\* \(2017\)](#)) and may reduce methanogenesis. Reducing NDF in the diet also affects the concentration and molar proportions of VFAs in the rumen. These changes lead to a balance away from acetate towards propionate as evidenced by reductions in acetate:propionate ratios resulting in a reduction in H<sub>2</sub> production due to utilisation for propionate formation. In this experiment, as with many forage-based studies ([Rira \*et al.\* \(2015\)](#); [Perry \*et al.\* \(2017\)](#); [Suybeng \*et al.\* \(2020, 2021\)](#)), the shift in the acetate:propionate ratio was small but consistent with the changes in NDF content of the diet.

Total VFA concentration increased with increasing leucaena inclusion in the diet. This was expected considering the improvement in diet quality resulting from leucaena



addition. Others have found decreases in VFA concentration through the addition of tanniniferous compounds in forages (Beauchemin *et al.* 2007; Rira *et al.* 2015). However, in the present study, it was apparent that the improvements in diet quality more than countered any potential negative effects of leucaena tannins on rumen fermentation, which have been shown to be minor (McSweeney *et al.* 1999). Apart from the change in acetate:propionate ratio, there were few effects on the molar proportions of individual VFAs, although a shift in fermentation from acetate to longer chain fatty acids was observed in cattle grazing leucaena (Tomkins *et al.* 2019). Other studies with leucaena have shown few effects on molar proportions of VFAs other than acetate and propionate (Rira *et al.* 2015; Montoya-Flores *et al.* 2020). In our experiment, pH responded quadratically to increasing leucaena in the diet, indicating a reduction in H<sup>+</sup> concentration in rumen fluid, particularly at the intermediate inclusion levels of leucaena. This was contrary to expectation based on acetate:propionate ratio and may have been influenced by elevated H<sub>2</sub> concentration (Ungerfeld 2020). By contrast, Rira *et al.* (2015) found that pH was reduced with leucaena feeding, and Montoya-Flores *et al.* (2020) observed no effects.

### Effect of the basal diet on methane response

The methane response to bioactives can also be influenced by the nature of the companion species in the diet. Aboagye *et al.* (2019) supplemented lucerne silage with different forms of hydrolysable tannin and showed small but variable reductions in methane yield (0–9%). In their study the quality of the companion forage, in this case lucerne silage, was very high (19.8% CP, 42.2% NDF). In the present study the companion forage was poor-quality Rhodes grass hay (5.1% CP, 73.1% NDF), and a much larger response in methane yield was observed. Suybeng *et al.* (2020) observed a reduction in methane yield when poor-quality Rhodes grass was fed with increasing proportions of the tannin-containing tropical legume *Desmanthus*. However, in a subsequent study where diets were isonitrogenous (10% CP, 68% NDF), by substituting *Desmanthus* with lucerne, no effect on methane yield was observed (Suybeng *et al.* 2021). In both studies, total phenolics (as catechin equivalents) were similar (1.2–2.3% DM).

In the main experiment, the CP content of the basal hay in the ration at only 5.1% was low and potentially insufficient to maintain optimal microbial activity in the rumen. Rumen ammonia concentration on the control diet was 3.7 mg/dL, which is below the generally accepted level for maximum microbial protein synthesis of 5 mL/dL (Satter and Slyter 1974). Increasing the CP of the diet through supplementation with leucaena increased both CP intake and rumen ammonia in a linear fashion. It can be concluded that increasing the availability of ammonia in the rumen would have increased microbial protein synthesis,

VFA concentration and DMI. In spite of the predicted increase in microbial protein synthesis, there was no concomitant increase in concentration of branch-chain fatty acids, which are derived from fermentation of protein (Keeney *et al.* 1962).

### Conclusions

The addition of leucaena to poor-quality tropical grass diets increased voluntary intake and reduced methane yield. The relationship between incremental levels of leucaena inclusion was described according to the equation:

$$\text{Methane yield (g/kg DMI)} = 19.8 - 0.09 \\ \times \text{leucaena percentage in the diet}$$

Although the relationship was linear, there was evidence that a 36% inclusion rate was optimal in terms of animal performance and methane emissions. The data suggest that the antimethanogenic effect of tannins, if completely neutralised, accounted for two-thirds of the methane reduction, with improved diet quality accounting for the other third. In parts of Queensland where leucaena can be successfully established, it can provide a reliable source of high-quality forage to supplement low-quality grass pastures and can also reduce methane emissions by up to 20%.

### Supplementary material

Supplementary material is available [online](#).

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**Data availability.** The data used to generate the results in the paper will be shared upon reasonable request to the corresponding author.

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