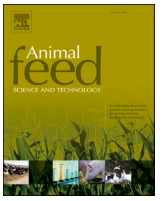
Animal Feed Science and Technology 305 (2023) 115777

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci



Evaluation of legumes for fermentability and protein fractions using *in vitro* rumen fermentation

Bereket Zeleke Tunkala [a,*], Kristy DiGiacomo [a], Pablo S. Alvarez Hess [b], Christopher P. Gardiner [c], Hafiz Suleria [a], Brian J. Leury [a], Frank R. Dunshea [a,d]

[a] *Faculty of Science, The University of Melbourne, Parkville 3010, VIC, Australia*
[b] *Agriculture Victoria Research, 1301 Hazeldean Road, Ellinbank, VIC 3821, Australia*
[c] *College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, QLD 4811, Australia*
[d] *Faculty of Biological Sciences, The University of Leeds, Leeds LS2 9JT, United Kingdom*

ARTICLE INFO

Keywords:
Soybean meal
Gas production
Methane
Ammonia-N
Alternative legumes
Condensed tannin

ABSTRACT

Diversifying feed with non-traditional options could minimize the dependency on traditional sources, maintain the feed supply throughout the year, and potentially reduce the cost of raising animals. A total of eight forage legumes including *Peltophorum pterocarpum, Neptunia monosperma, Vachellia sutherlandii* (Corkwood)*, Gliricidia sepium, Bauhinia hookeri* and three *Desmanthus* species (JCU4, JCU5 and JCU9) were collected to assess their *in vitro* fermentability, degradable and undegradable protein fractions using *in vitro* gas production method. Soybean meal and lucerne hay were used as control. The total gas production ranged from 12.8 mL/g in *P. pterocarpum* to 127.3 mL/g in soybean meal. The total volatile fatty acid (VFA) concentration from *G. sepium* (117.7 mM/L) and *V. sutherlandii* (111.3 mM/L) were larger than other legumes except for soybean meal (157.1 mM/L) and lucerne hay (130.4 mM/L), $P < 0.001$. The methane gas produced from *B. hookeri* and *P. pterocarpum* (0.39 and 0.32 mL/g) was lower than other feeds, $P < 0.001$. The *V. sutherlandii (*720 g/kg crude protein (CP)) and *G. sepium* (745 g/kg CP) had the greatest effective CP degradation (EPD) than other legume species examined, $P < 0.001$, which was approaching that measured in the control samples. The amount of protein fraction 'a' (rapidly degradable) was larger in JCU9 (551 g/kg CP), and *G. sepium* (472 g/kg CP), and lower in *B. hookeri* (10.9 g/kg CP) and *P. pterocarpum* (14.8 g/kg CP), $P < 0.001$. The *V. sutherlandii* (386 g/kg CP) and *G. sepium* (272 g/kg CP) exceeded other legumes in the proportion of fraction 'b' (slowly degradable), $P < 0.001$, but not the controls. The undegradable fraction increased with increasing phenolic content and reached more than 940 g/kg CP for both *B. hookeri* and *P. pterocarpum*. The *Desmanthus* cultivars showed intermediate values among the tested legumes in fermentation characteristics and shows potential to provide slowly degradable protein while reducing methane. The findings indicate the possibility of using *V. sutherlandii* and *G. sepium to* substitute other forages for their greater slowly degradable protein content. Moreover, *B. hookeri* and *P. pterocarpum* plants emerged as candidates to assist protein protection in the rumen and reduce methane emissions. However, these legumes need to be evaluated *in vivo* before promoting for further use to confirm the variability reported here.

Abbreviations: ADF, acid detergent fibre; CP, crude protein; DMD, dry matter digestibility; EPD, effective CP degradation; IVDP, *in vitro* degradable crude protein; ME, metabolizable energy; NDF, neutral detergent fibre; NFC, non fiber carbohydrate; OMD, organic matter digestibility; TCT, total condensed tannin; TDN, total digestible nutrient; TPC, total phenolic content; VFA, volatile fatty acids.

* Corresponding author.
E-mail address: btunkala@student.unimelb.edu.au (B.Z. Tunkala).

https://doi.org/10.1016/j.anifeedsci.2023.115777
Received 5 June 2023; Received in revised form 9 September 2023; Accepted 12 September 2023
Available online 14 September 2023

1. Introduction

The food-feed competition is a challenge faced by the livestock industry (Halmemies-Beauchet-Filleau et al., 2018). Moreover, the common protein supplements for ruminants are costly compared to other feed types (Wheeler and Reynolds, 2013). Climate change also risks the quality and availability of ruminant feed (Rojas-Downing et al., 2017). Therefore, demand has grown for alternative feeds with promising nutritional values and adaptability to climatic challenges (Abbeddou et al., 2011). Diversifying feed with non-traditional options could minimize the dependency on traditional sources, maintain the feed supply throughout the year, and potentially reduce the cost of raising animals.

Hundreds of legumes exist globally, but only a few are used as feed sources (Sońta and Rekiel, 2020). Forage legumes provide multiple phytochemicals with nutritional and health benefits for ruminants. However, legumes differ in chemical composition, protein fractions, ruminal degradability and the level of anti-nutritional contents that may affect rumen microbes, thereby limiting nutrient utilization in ruminant species (Makkar et al., 1995; Kaitho et al., 1998). These differences represent sources of variation between feed resources. Moreover, excessive rumen protein degradation in high-quality forage legumes could reduce their nutritional quality (Broderick, 1995) and result in high fecal and urinary N excretion (Wang et al., 2015). Therefore, these can also contribute to identifying suitable legumes as a replacement for conventional protein supplements.

This research focuses on underutilized legumes that grow in Australia's climate and northern tropical geographic regions. *Peltophorum pterocarpum* (syn *Peltophorum pterocarpum* (DC.) K. Heyne or Golden flame tree) is native to the Northern Territory of Australia and tropical Asia, a fast-growing tree, often used as a street/shade tree across a wide geographic area of northern Australia (ALA, null; Babu et al., 2016). *Neptunia monosperma* (Native sensitive plant) is a native Australian herbaceous legume with sensitive bipinnate leaves (ALA), occurs predominately in semiarid grassland regions of Northern and Western Australia (Bean, 2022), grows rapidly in the rainy season and dries out when the rain ceases (Holm and Eliot, 1980). *Vachellia sutherlandii (*syn *Acacia sutherlandii)* (Corkwood) is a native Australian shrub or tree, grows predominately in semiarid grassland regions of Northern and Western Queensland and into the Northern Territory and Western Australia (ALA).

Gliricidia sepium (Gliricidia) is a medium-sized and multipurpose use tree, utilized as animal forage in many parts of the tropical world for its high leaf production and quality (Simons and Stewart, 1994). *Desmanthus* species (Desmanthus) have nyctinastic bipinnate leaves, grow on the semi-arid regions across northern Australia, extensively grazed and well adapted to a wide range of land types (Gardiner, 2016). *Bauhinia hookeri* (Queensland Ebony) is a native Australian tree, predominately occurs in North East Queensland (ALA) and grow along drainage lines and banks of streams (Everist, 1986).

Evaluation of *in vitro* fermentability, methanogenic potential, and protein quality of feeds helps to determine the potential of feeds in fulfilling the nutritional requirement of ruminants and provides input into deciding the level of inclusion in a ration before *in vivo* experiments. Moreover, the condensed tannin in some legumes has the potential to mitigate methane emissions from ruminants (Tavendale et al., 2005; Lascano and Cárdenas, 2010), increase rumen by-pass proteins and enhance animal production efficiency (Jackson et al., 1996; Fondevila et al., 2002).

The *in vitro* fermentability of these plants yet to be evaluated except for a few studies evaluating *Gliricidia* (Edwards et al., 2012) and *Desmanthus* species (Durmic et al., 2017). To our knowledge, information on degradable protein fractions is also unavailable for all these species. Therefore, this experiment was conducted to evaluate the *in vitro* degradability, protein degradation and their relationship with phenolic content of eight legume species. The legumes selected in this experiment were expected to demonstrate different levels of *in vitro* fermentability, methanogenic potential and degradable protein fractions based on their chemical composition.

2. Material and methods

All procedures were conducted per the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHaMR,

Table 1
Summary of plant and sample types, harvesting date, location, soil type and temperature at each sampling place of legume forages tested *in vitro*.

Legumes	VS	BH	JCU4	JCU9	JCU5	GS	NMS	PP
Species								
Family	Leguminosae	Leguminosae	Leguminosae	Leguminosae	Leguminosae	Leguminosae	Leguminosae	Leguminosae
Sub-family	CSL	CDP	CSL	CSL	CSL	PPL	CSL	CSL
Harvesting details								
Plant habit	Tree	tree	shrub	shrub	shrub	shrub	forb	tree
Sample type	Leaf, buds	leaf	leaf	leaf	leaf	leaf	Leaf, fine stems	leaf
Sward height, m	1–4	4	1.5	1.5	1	3	0.5	5
Harvesting Date	20/10/21	25/11/21	7/10/21	7/10/21	7/10/21	25/11/21	26/11/21	25/11/21
Time	0900	0800	0830	0800	0900	0830	0800	0900
Location	Richmond	Townsville	Mareeba	Townsville	Mareeba	Townsville	Cloncurry	Townsville
Soil type	Vertosol	alluvial	Red clay loam	Loam	Red clay loam	Loam	Vertosol	Loam
Temp, max/min, ºC	33/17	30/25	30/16	30/24	30/16	30/24	38/23	30/24

VS: Vachelia sutherlandii; BH: Bauhinia hookeri; JCU4: Desmanthus bicornutus cv. JCU4; JCU9: Desmanthus pernambucanus JCU9; JCU5: Desmanthus virgatus JCU5; GS: Gliricidia sepium; NMS: Neptunia monosperma; PP: Peltophorum pterocarpum: CSL: Caesalpinioideae; CDP: Cercidoideae; PPL: Papilionideae;

2013). The Department of Jobs, Precincts and Regions Agricultural Research and Extension Animal Ethics Committee approved the preparation and use of cannulated cows from which rumen fluid was sourced for this experiment.

2.1. Substrates

Eight legumes (*V. sutherlandii, B. hookeri, D. bicornutus* cv. JCU4 (JCU4)*, D. pernambucanus* JCU9 (JCU9)*, D. virgatus* JCU5 (JCU5)*, G. sepium, N. monosperma,* and *P. pterocarpum)* plus two controls (lucerne hay and solvent-extracted soybean meal) were included as feed samples in this experiment (n = 10 feeds). The leaves of all legumes*,* buds *(V. sutherlandii)* and fine stems *(N. monosperma)* were harvested manually as presented in Table 1 from a variety of locations, and oven-dried for 48 h at 60ºC. All legumes were collected from North or Northwest Queensland which typically has a monsoonal climate with a hot humid wet season from December to March followed by long cool to mild dry season of six to seven months.

2.2. Collection and pre-incubation of rumen fluid

Two liters of rumen fluid was collected per run from four mid-lactation cannulated Holstein Friesian dairy cows at Agriculture Victoria (Ellinbank, Victoria) in the morning before feeding and transported to the laboratory as described by Tunkala et al. (2022). Cows were grazing perennial ryegrass (*Lollium perenne* L.) pasture, and wheat and barley grain mix (6 kg dry matter (DM) per day per cow) was supplied in the milking parlor. The rumen fluid was filtered using cheesecloth and pre-incubated for three hours using 10 g/L soluble sugars (3.33 g of maltose, 3.33 g of starch, and 3.33 g of xylose) to reduce the background ammonia-N before *in vitro* fermentation (Karlsson et al., 2009) in a 39ºC water bath (20-L Analogue Water bath, WB20, Ratek Instruments, Boronia, Australia). Sodium bicarbonate $NaHCO_3$ (3.1 g dissolved in 63 mL of McDougall's buffer per L of rumen fluid) was also added to the rumen fluid under continuous flushing of 8–10 psi carbon dioxide before pre-incubation and fermentation (ANKOM, 2018).

2.3. Fermentation and experimental design

The pre-incubated rumen fluid was mixed with McDougall's buffer to obtain a buffered rumen fluid with a 1:2 rumen fluid to buffer ratio. The feed samples were ground using grinder (Breville, The Coffee & Spice Grinder, Stainless Brushed Steel, Myer, Docklands, Australia) and sieved by 2 mm size sieve. A 500 mg sample of each substrate was weighed into 250 mL ANKOM bottles, and mixed with 90 mL buffered rumen fluid. Eight replications of each substrate were fermented *in vitro* using the ANKOM gas production system in three incubation runs for 24 h in a 39ºC water bath. Randomized complete block design (RCBD) was used, considering runs as a random experimental replicate.

The total number of bottles with ANKOM modules used per run were 42. Thirty modules were incubated for sample collection to analyse all parameters, and ammonia-N samples were collected from 10 modules in the first and second runs. The last run holds 20 modules for sampling all parameters and 20 exclusively for ammonia-N sampling. The remaining two modules per run were blanks and incubated as a background, making it a total of six.

2.4. Parameters measured

The feed chemical composition (crude protein (CP), acid detergent fibre (ADF), neutral detergent fibre (NDF), dry matter digestibility (DMD), organic matter digestibility (OMD), metabolizable energy (ME), fat, ash, non fiber carbohydrate (NFC), and total digestible nutrient (TDN)) was either measured or estimated in a commercial laboratory (FeedTest Laboratory, Agrifood Technology, Werribee, Australia) using near infrared spectroscopy. Total gas production was determined by the ANKOM gas production system. The N content of the unfermented substrates was quantified by the Kjeldahl system. The pH value in the rumen fluid was recorded post-fermentation using a pH meter (Oakton® Acorn™ series pH 6 m, Sigma-Aldrich, Castle Hill, Australia). Ammonia-N concentration was estimated by the colourimetric technique as described by Weatherburn (1967) using a multiscan colourimetric plate reader (Thermo Multiskan Spectrum, Thermo Fisher Scientific, Massachusetts, USA). The ammonia-N samples were collected from fermentation bottles by opening ANKOM modules and pipetted to 5 mL caped tubes at 0 h, 4 h, 8 h, 12 h, 16 h, and 24 h using separate modules for each hour per treatment and stored at − 20ºC until analysis.

The DMD was measured by pepsin-cellulase method (Dowman and Collins, 1982; AFFIA, 2014). The OMD and ME were determined using the equations from SCA (1990) and AFFIA (2014), respectively.

$$OMD = 6.83 + (0.847 * DMD) \quad (1)$$

$$ME = (0.203 * OMD) - 3.001 \quad (2)$$

The NFC and TDN were calculated using the following equations from Mertens (1997) and Linn and Martin (1989), respectively.

$$NFC = 100 - (CP + ash + fat + NDF) \quad (3)$$

$$TDN = 88.9 - (ADF * 0.779) \quad (4)$$

A 4 mL sample was collected for the volatile fatty acids (VFA) analysis to caped 5 mL tube from each module at the end of 24 h *in vitro* fermentation and kept frozen at − 20ºC till analysis. The VFAs, including acetic acid, propionic acid, isobutyric acid, butyric acid,

isovaleric acid, and valeric acid concentrations, were measured by gas chromatography fitted with a flame ionization detector using methyl valerate as the internal standard (Jouany, 1982).

Gas samples were also collected at the end of 24 h *in vitro* fermentation using the method described by Alvarez Hess et al. (2019) for methane analysis by gas chromatography (GC) (7890 A Agilent, Santa Clara, California, USA). Samples were analysed using Agilent 7890 A equipped with 3 detectors (TCD, μECD, FID), and GILSON GX-271 auto sampler for transferring the pressurised sample from Labco Exetainers® to the GC loops (1 mL × 2). The sample inlet and were GC loops flushed using helium between samples to avoid carryover. Columns used were: HayeSep® N 80/100 mesh, 0.5 m × 1/8 in, SST (precolumn for both channels); Porapak® QS 80/100 mesh, 2 m × 1/8 in. SST (analytical on TCD – FID channel); HayeSep® D, 80/100 mesh, 2 m × 1/8 in, SST (analytical to μECD).

The total phenolic (TPC) and condensed tannin contents (TCT) were extracted and estimated by using the methods of Feng et al. (2020) and Ali et al. (2021). Extracts were prepared using a 1/20 (w/v) sample-to-solvent ratio with 80% methanol in 1% formic acid, followed by incubation in an orbital shaker (ZWYR-240) at 150 rpm and 4ºC for 2 h. Extracts were centrifuged at 8000g for 20 min, and the supernatant was collected. The TPC was determined by mixing 25 μL extract, 25 μL Folin reagent solution, and 200 μL water in a 96-well plate (Costar, Corning, New York, USA), and the reaction mixture was incubated at room temperature in a dark room for 5 min. After that, 25 μL 10% (w:w) sodium carbonate was added, and the mixture was incubated again for 60 min at 25 °C. The TCT was measured by mixing 25 μL of sample solution with 150 μL of vanillin solution (4%). Subsequently, 25 μL of 32% H_2SO_4 was added to the mixture in 96-well plate. It was then incubated for 15 min in darkness at room temperature. The absorbance was measured using a spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for both and contents were expressed as DM percentage converted from mg of gallic acid equivalents per gram of sample (mg GAE/g).

The *in vitro* degradable CP (IVDP) was calculated from gas production and ammonia-N values at 4, 8, 12, 16 and 24 h incubation times using the following equation from Raab et al. (1983), as modified by Karlsson et al. (2009).

$$\text{IVDP} = \frac{\text{Ammonia} - \text{N at zero gas production}\left(b^0\text{intercept}\right) - \text{Ammonia} - \text{N in blank}}{\text{Total N of incubated feed}} \quad (5)$$

The proportion of protein fractions and the effective CP degradation value were estimated by fitting the non-linear equations of Ørskov and McDonald (1979) to the IVDP data using the exponential regression model of GenStat 21st edition.

$$Y = a + b * (1 - e^{-ct}) \quad (6)$$

Where: Y is the proportion of CP degraded at time t, 'a' is the proportion of CP degraded at time 0 h, 'b' is the proportion of slowly degradable CP, and 'c' is the degradation rate of fraction 'b'.

The effective CP degradation (EPD) value was calculated using the equation of Ørskov and McDonald (1979):

$$\text{EPD} = a + \frac{(b * c)}{(k + c)} \quad (7)$$

Where: the passage rate (k) was assumed to be 0.08 h^{-1}.

2.5. Statistical analysis

The substrate was used as a fixed effect to compute the differences between parameters using one-way ANOVA in GenStat 21st edition, except for IVDP and ammonia-N. The *in vitro* fermentation runs were used as random experimental replicates for all parameters. The single factor ANOVA model used was:

$$Y_{ij} = \mu + S_i + e_{ij}$$

Where: Y_i is the general mean of continuous dependent variables with $_j$ treatment replications ($_j = 8$), μ is the mean value of all substrates examined, and S_i is the fixed effect of each substrate (i = *V. sutherlandii, B. hookeri, D. bicornutus* cv. JCU4, *D. pernambucanus* JCU9, *D. virgatus* JCU5, *G. sepium,* Lucerne hay, *N. monosperma, P. pterocarpum, and* soybean meal) on the tested parameter, e_{ij} is the standard error term. The arithmetic mean values were compared using standard error of differences (SED) and considered statistically significant when $p < 0.05$.

The effects of the substrate and incubation time on the IVDP and ammonia-N values were evaluated by a two-way ANOVA using the model:

$$Y_{ijk} = \mu + S_i + T_j + ST_{ij} + e_{ijk}$$

Where: Y_{ijk}, μ, and S_i were described above, T_j is the fixed effect of time, ST_{ij} is the interaction effect between S and T, e_{ijk} is the standard error term of the k^{th} observation from the $(i, j)^{th}$ cell. The arithmetic mean values were compared using standard error of differences (SED) and considered statistically significant when $p < 0.05$.

The correlation matrix, exponential regression, and correlation coefficient between the total phenolic content values and the gas production, degradable protein fractions, methane, and total VFA values were determined using Pearson's correlation function and 2D scatter plot of Genstat 22nd Edition.

3. Results

3.1. Chemical composition

The chemical composition varied between legumes (Table 2); however, the results were not statistically analysed for lack of replications in the chemical composition data. The variation in CP ranged from 131 g/kg in *P. pterocarpum* to 233 g/kg in *G. sepium*. The lowest ADF was in *G. sepium*, followed by JCU9 and the maximum was detected in JCU4. The NDF content ranged from 255 g/kg in *G. sepium* to 43.6 g/kg in JCU4. The highest NFC was obtained from *P. pterocarpum*, and the lowest was from JCU4. The TDN of JCU9 numerically exceeded other legumes but not the control feeds.

The greatest TPC and TCT were observed in *B. hookeri*, *P. pterocarpum* and *N. monosperma* when compared with the other legume hays. The *Desmanthus* species showed TPC ranging from 41.3 to 46.1 g/kg and TCT from 11.2 to 16.8 g/kg. The TPC and TCT contents from *G. sepium* and *V. sutherlandii* were lower than other legume hays examined.

3.2. In vitro fermentation characteristics

The cumulative gas production, pH, gas composition (methane and carbon dioxide), and volatile fatty acids of legumes are shown in Table 3. The greatest total gas production, methane, and VFA concentrations occurred with the control samples, followed by *G. sepium, V. sutherlandii* and *Desmanthus* cultivars in decreasing order, $P < 0.001$. Among the tested legumes, the total gas production ranged from 12.8 mL/g DM in *P. pterocarpum* to 89.5 mL/g DM in *G. sepium*. There was no difference in the total gas production between the *Desmanthus* cultivars. The post-fermentation pH was not different between JCU4, *G. sepium* and *B. hookeri.*

The methane gas production and total VFA concentration were not different between *B. hookeri* and *P. pterocarpum*. The *B. hookeri* and *P. pterocarpum* showed lower methane gas production than other legumes tested, whereas the lowest VFA concentration was from *N. monosperma,* $P < 0.001$. The *Desmanthus* cultivars differed in methane emission, $P < 0.001$, but not in cumulative gas production and total VFA. The total VFA from *G. sepium* and *V. sutherlandii* was larger than that of the other legumes, $P < 0.001$, but not for the control samples.

3.3. Protein degradation

The ammonia-N volume decreased across fermentation time for most substrates evaluated except for *B. hookeri*, *P. pterocarpum* and *N. monosperma*, (Table 3). A greater ammonia-N concentration was recorded from the control feeds after 4 h incubation than other substrates, followed by *G. sepium* and *V. sutherlandii*, $P < 0.001$. The lowest ammonia-N was measured from *B. hookeri* and *P. pterocarpum* after 4 h incubation, $P < 0.001$.

The IVDP values were affected by the interaction between the substrates and fermentation time and increased with the fermentation time for all substrates, $P < 0.001$, as presented in Table 4. The maximum IVDP proportion was calculated for the control feeds, followed by *V. sutherlandii and G. sepium*, $P < 0.01$. The *B. hookeri* and *P. pterocarpum* showed a negative IVDP after 4 h incubation and reached 25% and 33% after 24 h, respectively, with $P < 0.01$. There was no difference between JCU4 and JCU5 across fermentation times regarding IVDP. The IVDP of JCU9 exceeded other *Desmanthus* cultivars after 24 h incubation, $P < 0.001$.

The amount of fraction 'a' was greater in JCU9 and *G. sepium*, and lower in *B. hookeri* and *P. pterocarpum* than other substrates, $P < 0.001$. The *V. sutherlandii* exceeded other experimental feeds in the proportion of fraction 'b' and degradation rate, followed by *G. sepium* and JCU5, $P < 0.001$. There was no difference between degradation rate of fraction 'b' in *V. sutherlandii and* lucerne hay. The degradation rate of fraction 'b' was lower in *B. hookeri* and *P. pterocarpum* than other samples, $P < 0.001$. The undegradable fraction

Table 2
Chemical composition of legume hays and soybean meal used in this experiment.

Parameters	VS	BH	JCU4	JCU9	JCU5	GS	LH	NMS	PP	SYM
Dry matter, g/kg	936	929	899	903	911	922	952	921	921	823
CP, g/kg	186	154	193	188	184	233	225	198	131	496
ADF, g/kg	214	220	252	139	174	136	246	186	221	122
NDF, g/kg	297	370	436	345	359	255	399	284	360	112
DMD, g/kg	631	427	598	736	660	707	651	547	329	935
OMD[1], g/kg	603	430	575	692	627	668	620	532	347	920
ME[1], MJ/kg DM	9.20	5.70	8.70	11.0	9.70	10.6	9.60	7.80	4.0	16.8
Fat, g/kg	55.0	29.0	42.0	53.0	50.0	53.0	41.0	29.0	27.0	120
Ash, g/kg	87.0	58.0	82.0	74.0	93.0	92.0	99.0	93.0	53.0	63.0
NFC[1], g/kg	375	389	247	340	314	367	237	396	429	318
TDN[1], g/kg	606	429	578	698	632	672	575	533	344	773
TPC, g/kg	7.30	157	46.1	41.3	44.9	10.8	3.00	130	153	1.20
TCT, g/kg	2.80	67.4	16.1	16.8	11.2	2.30	3.00	65.3	48.3	1.20

VS: Vachelia sutherlandii; BH: Bauhinia hookeri; JCU4: Desmanthus bicornutus cv. JCU4; JCU9: Desmanthus pernambucanus JCU9; JCU5: Desmanthus virgatus JCU5; GS: Gliricidia sepium; LH: Lucerne hay; NMS: Neptunia monosperma; PP: Peltophorum pterocarpum; SYM: soybean meal; 1 Calculated; ADF: Acid Detergent Fibre; NDF: Neutral Detergent Fibre; DMD: dry matter digestibility; OMD: Organic matter digestibility; ME: Metabolisable energy; NFC: Non fiber carbohydrate; TDN: Total digestible nutrient; TPC: Total phenolic content; TCT: Total condensed tannin

Table 3
The cumulative gas production (mL/g), pH, gas composition (methane and CO_2), ammonia-N (NH_3-N, μg/mL) and volatile fatty acids (VFA, mM/L) of legume hays and soybean meal after *in vitro* rumen fermentation for 24 h.

Variables	VS	BH	JCU4	JCU9	JCU5	GS	LH	NMS	PP	SYM	SED	Sign.[1]
Cumulative gas production, mL/g	83.0^{d}	20.0 ^{g}	60.8^{e}	58.0^{e}	55.4^{e}	89.5^{c}	101^{b}	32.1 ^{f}	12.8 ^{h}	127^{a}	5.52	* **
pH	6.71^{d}	6.76^{c}	6.74^{c}	6.84^{a}	6.79^{b}	6.76^{c}	6.65^{e}	6.79^{b}	6.81^{b}	6.74^{c}	0.022	* **
Methane, mL/g	2.71^{d}	0.39 ^{f}	2.27^{d}	2.74^{d}	1.55^{e}	5.03^{c}	6.49^{b}	1.38^{e}	0.32 ^{f}	10.3^{a}	0.540	* **
CO2, mL/g	80.3^{c}	19.6 ^{f}	58.5^{d}	55.3^{d}	54.9^{d}	84.4^{c}	94.6^{b}	30.7^{e}	12.5 ^{g}	117^{a}	5.91	* **
Total VFA, mM/L	111^{d}	97.0 ^{f}	108^{d}	103^{e}	103^{e}	118^{c}	130^{b}	79.5 ^{g}	96.9 ^{f}	157^{a}	3.40	* **
Acetic acid, %	42.3 ^{cd}	41.9^{d}	42.1^{d}	44.1^{b}	42.7^{c}	40.9^{e}	39.5 ^{f}	44.7^{a}	40.9^{e}	36.6 ^{g}	0.43	* **
Propionic acid, %	26.9^{b}	27.0^{b}	26.2^{c}	24.6^{d}	25.8^{c}	25.6^{c}	26.2^{c}	21.2 ^{f}	23.8^{e}	29.8^{a}	0.67	* **
Butyric acid, %	11.6^{d}	12.4^{c}	11.4^{de}	11.5^{de}	11.1^{e}	12.5^{c}	13.1^{b}	9.8	11.7^{d}	13.8^{a}	0.44	* **
Isobutyric acid, %	2.37^{c}	2.11^{d}	1.87^{e}	2.72^{b}	1.94^{e}	2.33^{c}	2.68^{b}	2.32^{c}	2.26^{c}	4.10^{a}	0.119	* **
Valeric, %	14.4^{c}	14.5^{c}	16.5^{b}	15.2^{bc}	16.3^{b}	16.5^{b}	15.9^{bc}	20.0^{a}	19.4^{a}	12.3^{d}	1.37	* **
Isovaleric, %	2.40^{c}	2.03 ^{f}	1.97 ^{g}	1.91	2.11^{e}	2.19^{d}	2.62^{b}	1.99^{fg}	2.03 ^{f}	3.49^{a}	0.040	* **
NH_3-$N_{4\ h}$, μg/mL	280^{b}	46.5 ^{h}	231^{d}	132^{e}	85.8 ^{f}	241 ^{cd}	249^{c}	85.9 ^{f}	61.5 ^{g}	331^{a}	14.89	* **
NH_3-$N_{8\ h}$, μg/mL	181^{b}	56.6 ^{f}	171^{b}	176^{b}	149^{c}	173^{b}	150^{c}	109^{d}	94.4^{e}	315^{a}	12.55	* **
NH_3-$N_{12\ h}$, μg/mL	133^{e}	73.2 ^{f}	131^{e}	200^{b}	150^{d}	186^{c}	142de	177^{c}	124^{e}	293^{a}	12.35	* **
NH_3-$N_{16\ h}$, μg/mL	108 ^{f}	89.2 ^{g}	90.0 ^{g}	127^{e}	138^{d}	142^{d}	133de	207^{b}	163^{c}	267^{a}	10.36	* **
NH_3-$N_{24\ h}$, μg/mL	79.8 ^{h}	110^{e}	98.1 ^{f}	88.5 ^{g}	127^{d}	66.8^{i}	74.4 ^{h}	223^{a}	189^{b}	168^{c}	7.33	* **

VS: Vachellia sutherlandii; BH: Bauhinia hookeri; JCU4: Desmanthus bicornutus cv. JCU4; JCU9: Desmanthus pernambucanus JCU9; JCU5: Desmanthus virgatus JCU5; GS: Gliricidia sepium; LH: Lucerne hay; NMS: Neptunia monosperma; PP: Peltophorum pterocarpum; SYM: Soybean meal; VFA: volatile fatty acids; 1Significance of of substrate effects; * ** , $P < 0.001$; The amount of methane and CO2 were calculated by converting their concentration ratio in the gas sample to their ratio in the volume of total gas production. Different superscript across each column stands for significant differences.

increased with increasing phenolic content and reached more than 940 g/kg for both *B. hookeri* and *P. pterocarpum*; therefore, it resulted in lowest EPD, $P < 0.001$. The EPD was greater in *G. sepium* than other tested substrates, followed by *V. sutherlandii*, $P < 0.001$.

3.4. Correlation between parameters

A correlation matrix between some of the analyzed and measured characteristics of the various legume hays and soybean meal shows strong relationships (Table 5). For example, total gas production was strongly and positively related to estimated ME ($R = 0.88$, $P = < 0.001$), calculated DMD ($R = 0.87$, $P < 0.001$), calculated OMD ($R = 0.88$, $P = < 0.001$), and TDN ($R = 0.82$, $P = < 0.01$), which indicates that it is related to the nutritional value of the feeds, although, of course, a correlation doesn't necessitate a cause-or-effect. On the other side, TPC was negatively associated with EPD ($R = 0.99$, $P = < 0.001$) and methane production ($R = -0.745$, $P = < 0.05$) (Table 5). Some of these relationships have been more fully explored by regression analysis.

The effect of total phenolic content on the values of total gas production, EPD, methane, and total VFA concentration are presented in Fig. 1. An exponential negative correlation was observed between the phenolic content of tested legumes and their fermentation characteristics. Overall, the increasing phenolic content resulted in decreased total gas ($R^2 = 0.96$), methane production ($R^2 = 0.86$), VFA concentration ($R^2 = 0.84$), and EPD ($R^2 = 0.37$). Likewise, the increasing condensed tannin content showed negative correlation with the *in vitro* fermentation characteristics examined in this study.

4. Discussion

These results demonstrated that *in vitro* fermentation characteristics, protein fractions (a, b and undegraded) and EPD were different between the legume species. The variation between legumes is likely driven by the high variability in the presence of secondary metabolites such as tannins and overall feed chemical composition, which can potentially influence *in vitro* fermentation values (Waghorn, 2008; Archimède et al., 2011). Therefore, the chemical constituent of substrates plays a significant role in fermentation parameters and protein fraction values of legumes. Another important finding is that the gas production values generated by *in vitro* fermentation system were highly correlated with estimates of digestibility and ME, providing confidence in the inferences drawn from the data.

4.1. Protein degradation

The *V. sutherlandii* and *G. sepium* had the greatest protein fraction 'a' and 'b' compared with the other legume species examined, which was approaching that measured in the control samples, indicating their potential to be used as a protein source for ruminants. The EPD of *V. sutherlandii* and *G. sepium* was not significantly different. However, they varied in terms of IVDP, total CP, fraction 'a' and 'b' contents, and degradation rate of fraction 'b'. Furthermore, the CP content of *N. monosperma* was 198 g/kg of the DM, which is in the same range as the CP content of the *Desmanthus* cultivars (184–193 g/kg DM). However, the EPD of *N. monosperma* was 160 g/kg CP (a = 76.0 g/kg CP and b = 85.6 g/kg CP), which is close to a quarter of the EPD content in *Desmanthus* cultivars. Therefore, this finding proves that the CP content of substrates may not ensure protein degradation during *in vitro* fermentation.

Table 4
The *in vitro* degradable crude protein (IVDP), protein fractions (a, b, undegraded), c (degradation rate, %/h) and effective crude protein degradation (EPD) of substrates after 24 h *in vitro* rumen fermentation.

IVDP	VS	BH	JCU4	JCU9	JCU5	GS	LH	NMS	PP	SYM	SED	Significance[1]		
Time, h												S	T	SxT
4	0.11^{d}	-0.22 ^{g}	0.12^{d}	0.12^{d}	0.13^{d}	0.08^{e}	0.29^{b}	0.25^{c}	-0.15 ^{f}	0.35^{a}	0.025	* *	* **	*
8	0.40^{c}	-0.10 ^{g}	0.15 ^{f}	0.27^{d}	0.22^{e}	0.22^{e}	0.39^{c}	0.45^{b}	0.13 ^{f}	0.63^{a}				
12	0.59^{b}	0.03 ^{h}	0.22 ^{g}	0.29 ^{f}	0.27 ^{f}	0.35^{e}	0.48^{c}	0.41^{d}	0.29 ^{f}	0.79^{a}				
16	0.67^{b}	0.20 ^{g}	0.27 ^{f}	0.30^{e}	0.28^{ef}	0.44^{d}	0.62^{c}	0.44^{d}	0.30^{e}	0.85^{a}				
24	0.73^{c}	0.25^{i}	0.33 ^{g}	0.40 ^{f}	0.30 ^{h}	0.58^{d}	0.85^{b}	0.48^{e}	0.33 ^{g}	0.91^{a}				
Fractions														
a, g/kg	334^{d}	10.9 ^{g}	386^{c}	451^{b}	324^{d}	472^{a}	449^{b}	76.0 ^{f}	14.8 ^{g}	185^{e}	12.22	* **		
b, g/kg	386^{b}	30.9 ^{h}	231^{d}	129 ^{f}	207^{e}	272^{c}	381^{b}	85.6 ^{g}	35.8 ^{h}	793^{a}				
Undegraded, g/kg	280 ^{f}	958^{a}	383^{e}	420^{d}	469^{c}	256 ^{g}	170 ^{h}	838^{b}	949^{a}	22.1^{i}				
c, %/h	1.57^{b}	0.13^{d}	0.96^{c}	0.12^{d}	0.86^{c}	1.13^{c}	1.59^{b}	0.36^{d}	0.15^{d}	3.30^{a}	0.298	* **		
EPD, g/kg	720^{d}	42.7^{i}	618^{e}	581 ^{f}	532 ^{g}	745^{c}	831^{b}	162 ^{h}	51.4^{i}	939^{a}	22.51	* **		

IVDP: In vitro protein degradability; VS: Vachelia sutherlandii; BH: Bauhinia hookeri; JCU4: Desmanthus bicornutus cv. JCU4; JCU9: Desmanthus pernambucanus JCU9; JCU5: Desmanthus virgatus JCU5; GS: Gliricidia sepium; LH: Lucerne hay; NMS: Neptunia monosperma; PP: Peltophorum pterocarpum; SYM: Soybean meal; EPD: effective CP degradation; 1Significance of effects of Substrates (S) x Time (T) and interactions; * , $P < 0.05$; * *, $P < 0.01$; * ** , $P < 0.001$; Different superscript across each column stands for significant differences.

Table 5
Correlation matrix between measured or estimated chemical composition of the legume hays and soyabean meal, and fermentation characteristics.

Gas production[a]	1	-										
Methane production[a]	2	0.93 * **	-									
Methane[a]	3	0.87 * **	0.95 * **	-								
Total[a] VFA	4	0.90 * **	0.94 * **	0.80 * *	-							
TPC[b]	5	-0.92 * **	-0.74 *	-0.70 *	-0.73 *	-						
TCT[b]	6	-0.85 * *	-0.67 *	-0.60	-0.73 *	0.97 * **	-					
ME[c]	7	0.87 * **	0.88 * **	0.87 * *	0.80 * *	-0.77 * *	-0.69 *					
DMD[d]	8	0.88 * **	0.84 * *	0.85 * *	0.74 *	-0.83 * *	-0.74 *	0.99 * **	-			
OMD[c]	9	0.88 * **	0.86 * *	0.86 * *	0.77 * *	-0.80 * *	-0.72 *	1.00 * **	1.00 * **	-		
TDN[d]	10	0.82 * *	0.73 *	0.76 * *	0.62	-0.84 * *	-0.75 *	0.94 * **	0.98 * **	0.96 * **	-	
CP[d]	11	0.78 * *	0.91 * **	0.85 * *	0.84 * *	-0.52	-0.45	0.89 * **	0.81 * *	0.86 * *	0.69 *	0.96 * **	-
EPD[a]	12	0.96 * **	0.83 * *	0.78 * *	0.81 * *	-0.99 * **	-0.95 * **	0.83 * *	0.87 * *	0.85 * *	0.85 * *	0.48	0.64 *
		1	2	3	4	5	6	7	8	9	10	11	12

[a] measured from *in vitro* fermentation characteristics;
[b] chemically determined;
[c] calculated from chemical components.
[d] determined by NIR and wet chemistry by a commercial laboratory;

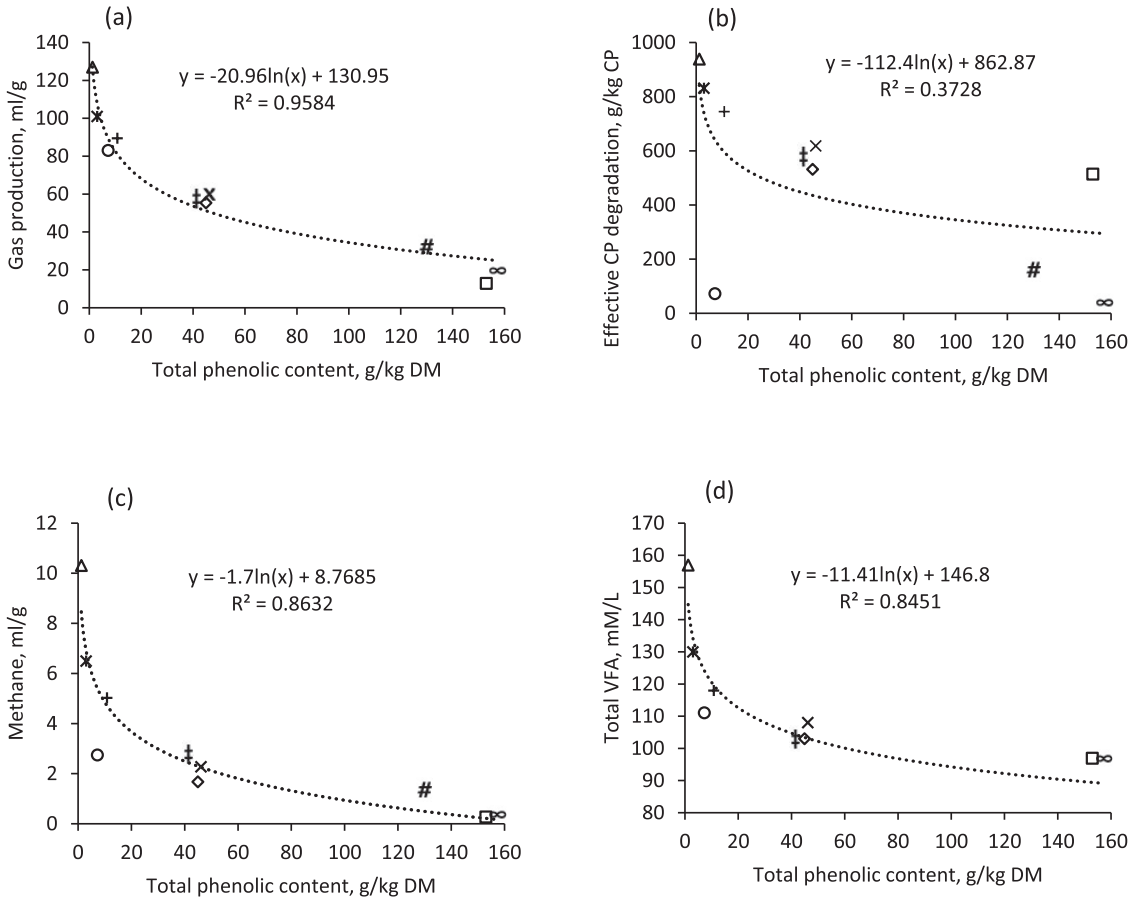


Fig. 1. The exponential regression equation, correlation curves and coefficients (R^2) between the total phenolic content in X axis and gas production (a), effective crude protein degradation (b), methane production (c) and total VFA (d) in Y axis for *Vachellia sutherlandii* (○), *Bauhinia hookeri* (∞), *Desmanthus bicornutus* cv. JCU4 (x), *Desmanthus pernambucanus* JCU9 (‡), *Desmanthus virgatus* JCU5 (◇), *Gliricidia sepium* (+), *Neptunia monosperma* (#), *Peltophorum pterocarpum* (□), Lucerne hay (*) and soybean meal (Δ) *in vitro* fermented using rumen fluid for 24 h. The p-values for the correlation between total phenolic content and the fermentation parameters were $P < 0.001$.

An inverse relationship demonstrated a reduction of EPD in legumes with increased phenolic content. This finding agrees with the study by Kapp-Bitter et al. (2020), who showed ammonia concentration was negatively related to the TPC and total tannin concentration following incubation of 35 temperate plants, including legumes for 24 h using the *in vitro* Hohenheim Gas Test method. This is likely the result of the protective effect of phenolic contents on EPD against rumen microorganisms responsible for the metabolism of N fractions by forming an unavailable tannin-protein complex (Bunglavan and Dutta, 2013). Mueller-Harvey (2006) has also reported an inverse relationship between ammonia and tannin contents in the feed after reviewing multiple reports from *in vitro* and *in vivo* studies. Therefore, the phenolic content is likely to determine the ammonia-N and protein values of fermented legumes.

The ammonia-N concentration was lower at 4 and 8 h of fermentation for *B. hookeri, P. pterocarpum*, and *N. monosperma,* contrary to other substrates. It increased slightly, showing the requirement of prolonged time for the breakdown of potentially degradable protein in these substrates. The interaction between the substrates and fermentation time on IVDP values could be related to the differences in the phenolic content of the legumes. This result is consistent with the research of Salman et al. (2022), who showed that a longer *in vitro* fermentation period reduced the total phenolic content of black tea, allowing more time for oxidation and breakdown of phenolic particles. Thus, despite the differences in the amount of IVDP, the IVDP of substrates with high TPC and TCT increases with the incubation time.

4.2. *In vitro fermentation characteristics*

The larger gas production from *G. sepium, V. sutherlandii* and *Desmanthus* cultivars than other experimental legumes could be positively related with their EPD protein content and negatively correlated with their tannin concentrations. However, prior reports on the *in vitro* fermentability and protein fractions of these legumes are scarce. The total gas production was similar to the report of Durmic et al. (2017) for *Desmanthus* cultivars collected in spring ranging from 72 to 77 mL/g. Moreover, the range of total gas production for soybean meal agrees with Faramarzi-Garmroodi et al. (2014) and lucerne hay with Aghajanzadeh-Golshani et al. (2015).

The gas production increases with the proportion of degradable protein as the availability of nitrogen fuels microbial activity (Cone et al., 2009; Karlsson et al., 2009). Furthermore, increased amount of tannin is proven to suppress gas production by bonding with digestible feed molecules, lowering enzymatic and microbial activities in feed degradation (Getachew et al., 2000; McSweeney et al., 2001). The variation in post-fermentation pH between legumes is also attributed to the differences in the degradable protein content as the degradation of protein to ammonia-N maintains high pH, and amino groups have a high buffering capacity (Jasaitis et al., 1987; Wadhwa et al., 2001). Therefore, our results confirm that the degradable protein (fraction a and b) content of the substrates directly affects gas production and post-fermentation pH values.

The lower volume of gas, methane, and VFA production from *B. hookeri, P. pterocarpum*, and *N. monosperma* resulted from the lower content of fractions 'a' and 'b', which cannot supply sufficient nitrogen for microorganisms during *in vitro* fermentation minimizing microbial proliferation and activity (Faramarzi-Garmroodi et al., 2014). Cone et al. (2009) and Karlsson et al. (2009) have demonstrated that nitrogen is a limiting factor for *in vitro* feed fermentation when adequate energy is available. Moreover, the lower methane production from *B. hookeri* and *P. pterocarpum* showed possession of potential anti-methanogenic characteristics. This finding supports the reports of Patra et al. (2006) and Puchala et al. (2012), who demonstrated that different tannin-containing feeds decreased methane production *in vitro* and *in vivo*. Tannin-containing feeds can modulate microbial composition through bactericidal effects and suppress microbial methanogenesis in the rumen fluid (Tavendale et al., 2005), which could justify the negative correlation observed between methane production and phenolic concentrations. Therefore, *B. hookeri* and *P. pterocarpum* could be exploited as a possible natural resource for methane mitigation.

The total VFA concentration in this experiment was greater than the results from Durmic et al. (2017) for *Desmanthus* cultivars, likely caused by the sugar added during pre-incubation and differences in harvesting seasons. However, the total VFA production decreased with decreasing gas production volume and increasing phenolic contents for all legumes. This finding agrees with Aderao et al. (2018), who reported that the production of VFA is directly related to the volume of gas production as fermentative gas is produced mainly when feedstuffs are fermented to acetic and butyric acids. This implies that factors affecting gas production, such as phenolic content and availability of EPD, have a similar impact on the total VFA concentration.

The *Desmanthus* cultivars were intermediate among the tested legumes in total gas production, EPD, methane production, total VFA values, and ammonia-N concentration, with values often around the inflection point of the relationships. Therefore, the *in vitro* fermentation parameters of *Desmanthus* plants show a dual benefit of these legumes with the potential to be fed to, or grazed by, ruminants as a degradable protein and energy source while reducing methane emissions. This could be attributed to the moderate amount of TPC and TCT available in *Desmanthus* cultivars which have protein protection and antimethanogenic characteristics (Patra et al., 2006; Bunglavan and Dutta, 2013). Moreover, studies showed that *Desmanthus* is adaptive from low to higher altitudes as a pasture legume, persists under heavy grazing, and is resilient to various environmental stresses such as drought, flood, and insect attacks (Pengelly and Conway, 2000; Gardiner and Swan, 2008; Gardiner, 2016). Therefore, *Desmanthus* may be able to impact more grazing animals than tree legumes as a pasture component.

Determination of feed fermentability and protein fractions using the *in vitro* gas production method is relatively time-saving and less costly compared to other *in vitro* methods (Tunkala et al., 2023 (under review)). Moreover, in the gas production system, IVDP is estimated *via* linear regression between gas production (as main variable) and ammonia nitrogen emission (as dependent variable) (Karlsson et al., 2009). It is assumed that the intercept of the regression shows the time that gas production was zero and no microbial protein synthesis has occurred, thus it represents ammonia-N produced due to feed degradation only. Furthermore, this method uses a mathematical approach to eliminate the confounding effects of de novo protein synthesis during fermentation (Bueno et al., 2005; Falahatizow et al., 2015). Therefore, EPD is derived from the outputs of this calculation and considers no microbial N contamination. However, the use of additional soluble sugar to minimize the background ammonia could be a limitation of this experiment resulting in a higher VFA production from Desmanthus cultivars compared with the report of Durmic et al. (2017).

The variations in fermentation characteristics and protein fractions between plants arise from differences in the plant species, varieties, harvesting season, and cultivation environments, such as soil quality (Bhardwaj and Hamama, 2012; Liebe et al., 2018). The differences between plants create an opportunity to select species and varieties based on their composition and fermentability. Generally, soybean meal and lucerne hay were higher in gas production, VFA, methane, fraction b protein, degradation rate, and EPD, followed by *V. sutherlandii* and *G. sepium* hays, which showed greater protein potential to be used as a substitute for control feeds. *Desmanthus* cultivars differed in methane, carbon dioxide, IVDP, and ammonia-N production while maintaining an intermediate position compared to tested legumes. The *B. hookeri* and *P. pterocarpum* hays were consistently lower in gas production, VFA, methane, protein fraction b, degradation rate, and EPD; higher in phenolic content and undegradable protein. Therefore, *B. hookeri* and *P. pterocarpum* emerged in this study as candidates to assist protein protection in the rumen and reduce methane emissions. *In vivo* experiments on these legumes could be worthy of confirming the variations and examining their post-rumen digestion and absorption.

5. Conclusion

Despite legumes showing potential for use as protein sources and methane-mitigating feeds, none of the legume hays examined in this experiment exceeded the total VFA, protein fraction 'b' and EPD of the lucerne hay. The *V. sutherlandii* and *G. sepium hays* were superior to other legumes based on fraction 'b' protein potential and could be used as a substitute for control feeds used in this study. The *B. hookeri* and *P. pterocarpum* plants could assist protein protection in the rumen and reduce methane emissions. The *Desmanthus* cultivars were intermediate among the tested legumes in fermentation characteristics and possess a dual benefit with the potential to supply an effectively degradable protein while reducing methane emissions. The findings indicate the possibility of selecting legumes for their feed value based on *in vitro* fermentation characteristics and protein quality; however, these legumes need to be tested *in vivo*

before being promoted for further use to confirm the variability reported here.

CRediT authorship contribution statement

B. Z. Tunkala: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Project administration; **P. S. Alvarez Hess**: Supervision, Writing – review & editing. **K. DiGiacomo**: Funding acquisition, Supervision, Writing – review & editing; **C. P. Gardiner:** Resources, Writing – review & editing. **H. Suleria**: Methodology. **B. J. Leury**: Methodology, Funding acquisition, Supervision, Writing – review & editing. **F. R. Dunshea**: Conceptualization, Methodology, Data curation, Formal analysis, Supervision, Writing – review & editing. All authors have read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

The authors are grateful to DJPR Ellinbank farm management and staff for assistance with rumen fluid collection. This work was supported by the Melbourne Research Scholarship for graduate study in the University of Melbourne. Legumes were supplied by Agrimix, Queensland.

References

Abbeddou, S., Riwahi, S., Iniguez, L., Zaklouta, M., Hess, H., Kreuzer, M., 2011. Ruminal degradability, digestibility, energy content, and influence on nitrogen turnover of various Mediterranean by-products in fat-tailed Awassi sheep. Anim. Feed Sci. Technol. 163, 99–110.

Aderao, G.N., Sahoo, A., Bhatt, R.S., Kumawat, P.K., Soni, L., 2018. In vitro rumen fermentation kinetics, metabolite production, methane and substrate degradability of polyphenol rich plant leaves and their component complete feed blocks. J. Anim. Sci. Technol. 60, 26.

AFFIA, 2014. Australian Fodder Industry Association - Laboratory Methods Manual. Melbourn, VIC, Australia.

Aghajanzadeh-Golshani, A., Maheri-Sis, N., Salamat Doust-Nobar, R., Ebrahimnezhad, Y., Ghorbani, A., 2015. Developing a modified in vitro gas production technique to replace the nylon bag method of evaluating protein degradation of alfalfa hay in ruminants. Iran. J. Appl. Anim. Sci. 5, 339–345.

ALA, Atlass of Living Australia. Available online: ⟨https://www.ala.org.au⟩ Accessed on 02 June 2023.

Ali, A., Wu, H., Ponnampalam, E.N., Cottrell, J.J., Dunshea, F.R., Suleria, H.A., 2021. Comprehensive profiling of most widely used spices for their phenolic compounds through LC-ESI-QTOF-MS2 and their antioxidant potential. Antioxidants 10, 721.

Alvarez Hess, P.S., Eckard, R.J., Jacobs, J.L., Hannah, M.C., Moate, P.J., 2019. Comparison of five methods for the estimation of methane production from vented in vitro systems. J. Sci. Food Agric. 99, 109–116.

ANKOM, 2018. ANKOM Gas Production system operator's manual.

Archimède, H., Eugène, M., Magdeleine, C.M., Boval, M., Martin, C., Morgavi, D., Lecomte, P., Doreau, M., 2011. Comparison of methane production between C3 and C4 grasses and legumes. Anim. Feed Sci. Technol. 166, 59–64.

Babu, M., Anand, A., Hakkim, F., Haq, Q., 2016. A review on phyto pharmacological aspects of Peltophorum pterocarpum (DC) Baker Ex. K. Heyne. Inter. J. Adv. Res 4, 801–807.

Bean, A., 2022. A revision of Neptunia Lour.(Leguminosae: subfamily Caesalpinioideae, Mimosoid clade) in Australia and Malesia.

Bhardwaj, H.L., Hamama, A.A., 2012. Cultivar and growing location effects on white lupin immature green seeds. J. Agric. Sci. 4, 135.

Broderick, G.A., 1995. Desirable characteristics of forage legumes for improving protein utilization in ruminants. J. Anim. Sci. 73, 2760–2773.

Bueno, I.C., Cabral Filho, S.L., Gobbo, S.P., Louvandini, H., Vitti, D.M., Abdalla, A.L., 2005. Influence of inoculum source in a gas production method. Anim. Feed Sci. Technol. 123, 95–105.

Bunglavan, S., Dutta, N., 2013. Use of tannins as organic protectants of proteins in digestion of ruminants. J. Livest. Sci. 4, 67–77.

Cone, J., Rodrigues, M., Guedes, C., Blok, M., 2009. Comparison of protein fermentation characteristics in rumen fluid determined with the gas production technique and the nylon bag technique. Anim. Feed Sci. Technol. 153, 28–38.

Dowman, M.G., Collins, F.C., 1982. The use of enzymes to predict the digestibility of animal feeds. J. Sci. Food Agric. 33, 689–696.

Durmic, Z., Ramírez-Restrepo, C.A., Gardiner, C., O'Neill, C.J., Hussein, E., Vercoe, P.E., 2017. Differences in the nutrient concentrations, in vitro methanogenic potential and other fermentative traits of tropical grasses and legumes for beef production systems in northern Australia. J. Sci. Food Agric. 97, 4075–4086.

Edwards, A., MLambo, V., Lallo, C., Garcia, G., 2012. Yield, chemical composition and in vitro ruminal fermentation of the leaves of Leucaena leucocephala, Gliricidia sepium and Trichanthera gigantea as influenced by harvesting frequency. J. Anim. Sci. Adv. 2, 321–331.

Everist, S.L., 1986. Use of fodder trees and shrubs. Use of fodder trees and shrubs.

Falahatizow, J., Danesh Mesgaran, M., Vakili, A.R., Tahmasbi, A.M., Nazari, M.R., 2015. The estimation of ruminal protein degradation parameters of various feeds using in vitro modified gas production technique. Iran. J. Vet. Res 16, 47–52.

Faramarzi-Garmroodi, A., Mesgaran, M.D., Vakili, A., Hodjatpanah-montazeri, A., Manshadi, M., 2014. In vitro rumen fermentation characteristics of intact or oil free of various protein sources. Annu. Res. Rev. Biol. 500–508.

Feng, Y., Dunshea, F.R., Suleria, H.A., 2020. LC-ESI-QTOF/MS characterization of bioactive compounds from black spices and their potential antioxidant activities. J. Food Sci. Technol. 57, 4671–4687.

Fondevila, M., Nogueira-Filho, J., Barrios-Urdaneta, A., 2002. In vitro microbial fermentation and protein utilisation of tropical forage legumes grown during the dry season. Anim. Feed Sci. Technol. 95, 1–14.

Gardiner, C., 2016. Developing and commercializing new pasture legumes for clay soils in the semi-arid rangelands of northern Australia: the new Desmanthus cultivars JCU 1-5 and the Progardes story. Tropical forage legumes: harnessing the potential of Desmanthus and other genera for heavy clay soils. CABI Wallingford, UK, pp. 283–304.

Gardiner, C., Swan, S., 2008. Abandoned pasture legumes offer potential economic and environmental benefits in semiarid clay soil rangelands.

Getachew, G., Makkar, H., Becker, K., 2000. Effect of polyethylene glycol on in vitro degradability ofnitrogen and microbial protein synthesis fromtannin-rich browse and herbaceous legumes. Br. J. Nutr. 84, 73–83.

Halmemies-Beauchet-Filleau, A., Rinne, M., Lamminen, M., Mapato, C., Ampapon, T., Wanapat, M., Vanhatalo, A., 2018. Alternative and novel feeds for ruminants: Nutritive value, product quality and environmental aspects. Animal 12, s295–s309.

Holm, A.M., Eliot, G., 1980. Seasonal changes in the nutritive value of some native pasture species in north-western Australia. Rangel. J. 2, 175–182.

Jackson, F.S., Barry, T.N., Lascano, C., Palmer, B., 1996. The extractable and bound condensed tannin content of leaves from tropical tree, shrub and forage legumes. J. Sci. Food Agric. 71, 103–110.

Jasaitis, D., Wohlt, J., Evans, J., 1987. Influence of feed ion content on buffering capacity of ruminant feedstuffs in vitro. J. Dairy Sci. 70, 1391–1403.

Jouany, J., 1982. Volatile fatty acid and alcohol determination in digestive contents, silage juices, bacterial cultures and anaerobic fermentor contents.
Kaitho, R., Umunna, N., Nsahlai, I., Tamminga, S., Van Bruchem, J., 1998. Digestibility of rumen undegradable protein from 40 browse species measured by mobile nylon bag and in vitro techniques. BSAP Occas. Publ. 22, 123–126.
Kapp-Bitter, A.N., Dickhoefer, U., Kreuzer, M., Leiber, F., 2020. Mature herbs as supplements to ruminant diets: effects on in vitro ruminal fermentation and ammonia production. Anim. Prod. Sci. 61, 470–479.
Karlsson, L., Hetta, M., Udén, P., Martinsson, K., 2009. New methodology for estimating rumen protein degradation using the in vitro gas production technique. Anim. Feed Sci. Technol. 153, 193–202.
Lascano, C.E., Cárdenas, E., 2010. Alternatives for methane emission mitigation in livestock systems. Rev. Bras. De. Zootec. 39, 175–182.
Liebe, D.M., Firkins, J.L., Tran, H., Kononoff, P.J., White, R.R., 2018. Technical note: Methodological and feed factors affecting measurement of protein A, B, and C fractions, degradation rate, and intestinal digestibility of rumen-undegraded protein. J. Dairy Sci. 101, 8046–8053.
Linn, J., Martin, N., 1989. Forage quality tests and interpretation. Minnesota EXT. Service, AG-FO-2637. University of Minnesota, Saint Paul.
Makkar, H.P., Blümmel, M., Becker, K., 1995. In vitro effects of and interactions between tannins and saponins and fate of tannins in the rumen. J. Sci. Food Agric. 69, 481–493.
McSweeney, C., Palmer, B., McNeill, D., Krause, D., 2001. Microbial interactions with tannins: nutritional consequences for ruminants. Anim. Feed Sci. Technol. 91, 83–93.
Mertens, D., 1997. Creating a system for meeting the fiber requirements of dairy cows. J. Dairy Sci. 80, 1463–1481.
Mueller-Harvey, I., 2006. Unravelling the conundrum of tannins in animal nutrition and health. J. Sci. Food Agric. 86, 2010–2037.
NHaMR, C., 2013. Australian code for the care and use of animals for scientific purposes. National Health and Medical Research Council, Canberra.
Ørskov, E., McDonald, I., 1979. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. J. Agric. Sci. 92, 499–503.
Patra, A., Kamra, D., Agarwal, N., 2006. Effect of plant extracts on in vitro methanogenesis, enzyme activities and fermentation of feed in rumen liquor of buffalo. Anim. Feed Sci. Technol. 128, 276–291.
Pengelly, B.C., Conway, M.J., 2000. Pastures on cropping soils: which tropical pasture legume to use? Trop. Grassl. 34, 162–168.
Puchala, R., Animut, G., Patra, A., Detweiler, G., Wells, J., Varel, V., Sahlu, T., Goetsch, A., 2012. Methane emissions by goats consuming Sericea lespedeza at different feeding frequencies. Anim. Feed Sci. Technol. 175, 76–84.
Raab, L., Cafantaris, B., Jilg, T., Menke, K., 1983. Rumen protein degradation and biosynthesis: 1. A new method for determination of protein degradation in rumen fluid in vitro. Br. J. Nutr. 50, 569–582.
Rojas-Downing, M.M., Nejadhashemi, A.P., Harrigan, T., Woznicki, S.A., 2017. Climate change and livestock: Impacts, adaptation, and mitigation. Clim. Risk Manag. 16, 145–163.
Salman, S., Öz, G., Felek, R., Haznedar, A., Turna, T., Özdemir, F., 2022. Effects of fermentation time on phenolic composition, antioxidant and antimicrobial activities of green, oolong, and black teas. Food Biosci. 49, 101884.
SCA, 1990. Feeding standards for Australian livestock. Ruminants. Csiro Publishing, Melbourne.
Simons, A., Stewart, J., 1994. 2.2 Gliricidia sepium-a Multipurpose Forage Tree Legume. semanticscholar. org.
Sońta, M., Rekiel, A., 2020. Legumes–use for nutritional and feeding purposes. J. Elem.
Tavendale, M.H., Meagher, L.P., Pacheco, D., Walker, N., Attwood, G.T., Sivakumaran, S., 2005. Methane production from in vitro rumen incubations with Lotus pedunculatus and Medicago sativa, and effects of extractable condensed tannin fractions on methanogenesis. Anim. Feed Sci. Technol. 123, 403–419.
Tunkala, B.Z., DiGiacomo, K., Hess, P.S.A., Dunshea, F.R., Leury, B.J., 2022. Rumen fluid preservation for in vitro gas production systems. Anim. Feed Sci. Technol., 115405
Tunkala, B.Z., DiGiacomo, K., Alvarez Hess, P.S., Dunshea, F.R., Leury, B.J., 2023. (under review). In vitro protein fractionation methods for ruminant feeds. Animal: The international journal of animal biosciences.
Wadhwa, D., Beck, N., Borgida, L., Dhanoa, M., Dewhurst, R.J., 2001. Development of a simple in vitro assay for estimating net rumen acid load from diet ingredients. J. Dairy Sci. 84, 1109–1117.
Waghorn, G., 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production—progress and challenges. Anim. Feed Sci. Technol. 147, 116–139.
Wang, Y., McAllister, T.A., Acharya, S., 2015. Condensed tannins in sainfoin: composition, concentration, and effects on nutritive and feeding value of sainfoin forage. Crop Sci. 55, 13–22.
Weatherburn, M., 1967. Phenol-hypochlorite reaction for determination of ammonia. Anal. Chem. 39, 971–974.
Wheeler, T., Reynolds, C., 2013. Predicting the risks from climate change to forage and crop production for animal feed. Anim. Front. 3, 36–41.