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Antimethanogenic bioactivity of Australian plants for grazing systems

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Table of Contents

Abstract

We investigated the 'antimethanogenic' bioactivity of a range of fodder plants, plant extracts and selected feed additives. We also investigated the variability, persistency and mechanisms behind this bioactivity, using a lab-based system. We identified a range of tropical legumes (e.g. *Leucaena leucocephala, Desmanthus virgatus*), novel forages (e.g. turnip and chicory), plant extracts (e.g. *Eremophila glabra, Santalum spicatum*) and feed additives (e.g. grape marc or marine products – DHA and *Nannochloropsis oculata*), that have the potential to reduce methane in the rumen. We also demonstrated that, under laboratory conditions, one plant (*E. glabra*), reduced methane by directly affecting the methanogens in the rumen. This effect persisted over several weeks and we are now conducting a study to confirm it works in sheep. We purified specific fractions that are responsible for these effects, which will help lead us to the specific compounds that are antimethanogenic and the mechanism behind their action. Most variability in anitmethanogenic bioactivity was observed when plants were grown at different locations and between different plant accessions within a species. Season, phenology and grazing had less influence on the variability. Our results will assist in developing new grazing and management systems for reducing methane emissions from grazing ruminants.

Executive summary
There is global interest in screening both commonly used and novel plants for their potential to reduce methane emissions from livestock. Characterising the variability of the antimethanogenic effects of these plants and identifying the bioactive compound(s) and the mechanism(s) responsible for the effect will assist in developing practical on-farm applications for reducing methane emissions from the ruminants. The aim of this project was to investigate a range of fodder plants for their potential to reduce methane emissions from livestock. Our specific objectives were to:

1. Screen selected Australian native perennial shrub species, tropical legumes and forages from northern Queensland and alternative forages and supplements from southern Australia for anti-methanogenic properties
2. Characterise the variability in anti-methanogenic activity in plant materials as influenced by plant collection site, phenological stage and season.
3. Identify possible mechanism(s) and persistency of anti-methanogenic actions from active plants and their compounds.
4. Isolate and identify bioactive compound(s) with antimethanogenic properties from candidate plants and plant materials, and
5. Validate *in vitro* findings *in vivo*

Our approach in this study was to build on a database of information about beneficial bioactive properties of 130 species of Australian native perennial plants adapted to the low-medium rainfall zones of Australia. We extended this research by screening commonly used fodder plants, plant extracts and selected feed additives and grazing plants that are, or could be, used in wider livestock production zones particularly those suitable for use in northern Australia. We used both batch and continuous culture (RUSITEC) *in vitro* fermentation systems to screen and identify the most promising antimethanogenic candidates. The batch system was used mainly to measure the variability of the anti-methanogenic activity of plants as a function of plant collection site, phenology, season and response to grazing. The continuous culture RUSITEC system was valuable for testing the persistency of the antimethanogenic effects of the most promising candidates, as well as providing some insight into their mechanisms of action by studying their effects on the microbial ecology. We also used pure cultures of methanogen species to provide insight into the mechanisms of action by testing whether extracts had broad or specific activity against methanogens and other ruminal microorganisms. Initially crude extracts were tested for antimethanogenic effects to identify the most promising candidate. Extracts from the most promising plant were fractionated using solvent gradients and then purified further using High Performance Liquid Chromatography (HPLC). These fractions were tested in the *in vitro* batch system to help identify the fractions that contained the compounds responsible for the antimethanogenic effects. The most promising antimethanogenic plant was taken through from *in vitro* screening to an *in vivo* experiment to establish whether the results from the laboratory occur when tested in animals.

We identified a range of tropical legumes (e.g. *Leucaena leucocephala, Desmanthus virgatus*), novel forages (e.g. turnip and chicory), plant extracts (e.g. *Eremophila glabra, Santalum spicatum*) and feed additives (e.g. grape marc or marine products – DHA and *Nannochloropsis oculata*), that have the potential to reduce methane in the rumen. Variability was assessed in a sub-set of plant species with the most potential to reduce methane in grazing systems. The most variability was observed when plants were grown

at different locations and between different plant accessions within a species. Season, phenology and grazing appeared to have less influence on the variability.

When we used pure cultures to gain an understanding of how some of these plants may be affecting methane production we found a mixture of broad and specific antimicrobial activities. Ethanolic extracts from 12 plants were tested against pure cultures of four representative rumen methanogenic species: *Methanobrevibacter gottschalkii, M.ruminantium, Methanobacterium bryantii* and *Methanosarcina barkerii.* In some extracts the effect was broad (i.e. 100% inhibition of all methanogens tested), while in others partial and specific effects were observed (limited reduction, only some methanogens affected). The mixture of broad and specific action of different plants provides evidence that there are different compounds and different mechanisms involved in the inhibition. Analysis of these effects at the chemical and molecular level may provide an insight into key differences between methanogen species that could be targeted for manipulation. The wide spectrum of activities also indicates that a combination of plants integrated into a more diverse grazing system will be effective against a range of different methanogens.

E. glabra was one of the most potent antimethanogenic plants and was pursued in most detail in this project. Ethanolic extracts of this plant caused up to a 50% reduction in methane production in *in vitro* batch fermentation and also inhibited 80%-100% of the growth of selected rumen methanogens when tested in pure culture. The effect of the extract did not diminish after adding PEG (tannin binder), implying that the antimethanogenic effect in this plant is likely to be related to compounds other than tannins. Although serrulatane diterpenes were purified from *E. glabra* and are known to be bioactive, they did not cause inhibition of methane production *in vitro*. Instead there were 3 other fractions that contain the compounds responsible for the antimethanogenic effects, which need to be investigated further. We also demonstrated that, in continuous culture, the effects of *E. glabra* are dose dependent. At higher doses there seems to be a general inhibition of rumen fermentation but at lower doses the effects are more specific. This was supported by molecular microbial ecology studies on samples that were taken during continuous culture, which suggested that at low inclusion (15%) *E. glabra* reduced methane by directly affecting the methanogens in the rumen. At higher rates of inclusion (25% and 40%) there was a more general effect on overall fermentation, There was some evidence that *F. succinogenes* was inhibited at this inclusion rate but there were no indications from the fermentation end-product that this had caused any negative effects. The effects of *E. glabra* on methane production persisted over several weeks, and we are now conducting a study to confirm this effect in sheep. We will feed *E. glabra* at 15% of the total diet to sheep in the animal house and measure their methane emissions in respiration chambers. If the in vitro results are validated in vivo, the specific bioactive effect of *E. glabra* may provide a viable pathway to develop strategies for long-term methane reduction in grazing animals.

Our results are valuable to researchers trying to understand the mechanisms behind factors that reduce methane production in the rumen and what happens more generally to the pathways of rumen fermentation. However, they also benefit producers because the plants that have been screened in this study are known to be grazed by livestock and in many cases are currently being used in grazing systems or have been planted in experimental grazing plots around Australia. The new knowledge that has been generated in this project provides evidence to producers that they are not limited by

choice of the species and accessions that could make up their pasture base and reduce their emissions. Some of those choices will need to be coupled with changes in the grazing management of the livestock to take full advantage of the opportunity to reduce emissions.

Background:
There is global interest in screening both commonly used and novel plants for their potential to reduce methane emissions from livestock. Identifying the bioactive compound(s), the variability and the mechanism(s) responsible for this antimethanogenic effect will assist in developing practical on-farm applications for reducing methane emissions from the ruminants. The aim of this project was to investigate the bioactivity of a range of fodder plants for their potential to reduce methane emissions from livestock and to investigate the most promising candidates in more detail. Our specific objectives were to:

1. Screen selected Australian native perennial shrub species, tropical legumes and forages from northern Queensland and alternative forages and supplements from southern Australia for anti-methanogenic properties
2. Characterise the variability in anti-methanogenic activity in plant materials as influenced by plant collection site, phenological stage and season.
3. Identify possible mechanism(s) and persistency of anti-methanogenic actions from active plants and their compounds.
4. Isolate and identify bioactive compound(s) with antimethanogenic properties from candidate plants and plant materials, and
5. Validate *in vitro* findings *in vivo*

This project built on a database of information about beneficial bioactive properties of 130 species of Australian native perennial plants adapted to the low-medium rainfall zones of Australia. We extended this research by screening commonly used fodder plants, plant extracts and selected feed additives and grazing plants that are, or could be, used in wider livestock production zones, particularly those suitable for use in northern Australia. A core part of the project was the access to experimental plots of a large number of species and accessions of novel plants as well as feed additives being used in feeding and respiration chamber experiments and new collections of plants from around Australia. The batch system was used mainly to measure the variability of the anti-methanogenic activity of plants as a function of plant collection site, phenology, season and response to grazing. The continuous culture RUSITEC system was valuable for testing the persistency of the antimethanogenic effects of the most promising candidates, as well as providing some insight into their mechanisms of action by studying the effects on the microbial ecology. We also used pure cultures of methanogen species to provide some insight into the mechanisms of action by testing whether extracts had broad or specific activity against methanogens and other ruminal microorganisms. Major fractions containing secondary compounds were purified from extracts of the most promising plant and tested in the *in vitro* batch system to narrow down the identity of the compounds responsible for the antimethanogenic effects. The serrulatane diterpenes were the only specific compounds purified from *E. glabra* because they were known to have antimicrobial properties. By the completion of this project one of the most promising antimethanogenic plants (*E. glabra*) will be taken through from *in vitro* screening to an *in vivo* experiment to establish whether the results from the laboratory represent what occurs when the plant is fed to animals.

This report is divided into 8 sections. The first 5 sections relate directly to the 5 objectives listed above which have been addressed in the form of at least one paper. The paper provide an introduction, the specific methods used, results and discussion. There are additional sections on the papers accepted for publication in peer review

journals, media coverage and the integration we've had with other projects within the RELRP program.

Section 1. Screen selected Australian native perennial shrub species, tropical legumes and forages from northern Queensland and alternative forages and supplements from southern Australia for anti-methanogenic properties (4 draft papers provided)

Draft paper 1 *- Draft manuscript – short communication*

In vitro fermentative traits of selected forages in North Queensland

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Abstract

The aim of this study was to examine selected tropical pastures to identify variability in the *in vitro* fermentative traits amongst these. Grass samples (*n* = 26) and legume samples (*n* = 5) were analysed in an *in vitro* batch fermentation system. *In vitro* fermentation parameters varied between different plant species, with microbial gas production ranging from 44 kPa (*Astrebla sp*. TV0021) to 93 kPa (*Leucaena leucocephala* cv.) and VFA from 81 mmol/L (*Heteropogon contortus* TV0002) to 107 mmol/L (*Leucaena leucocephala* cv.). Methane values ranged from 29 mL/g DM (*Astrebla sp*.) to 60 mL/g (*Stylosanthes seabrana*). Seven grasses and one legume reduced methane significantly when compared to the respective controls, and only two plants, *Chloris gayana* TV0010 and *Cenchrus ciliaris* TV007 did not reduce gas and VFA. It was concluded that different quality and fermentation profiles provide opportunities for productive grazing on these pastures, while offering some methane mitigation options in the context of northern Australian farming production systems. However, studies are needed to confirm these findings *in vivo*.

Introduction

Livestock production systems in Australia are diverse and browse plants play an important role in providing feed for ruminants. However, consuming high fibre diets is associated with relatively high methane production during microbial fermentation of these feeds in the rumen (Beijer, 1952; Beauchemin et al., 2008). There is evidence of variability in rumen methanogenic potential amongst forage plants (Waghorn et al., 2002; Tavendale et al., 2005; Soliva et al., 2008). More recently, screening of plants targeting summer-active perennials for low rainfall areas revealed that similar variability may exist in forage plants in Australia (Durmic et al., 2010). Results from this initial screening implied that there is great diversity amongst the plants in terms of their fermentative properties, and that it is possible to select plants that support fermentation, but have relatively low methane production when fermented in the rumen. These results generated interest to expand the list of plants that were being screened to include plants that are of particular interest to northern Queensland and extensive beef

production. The aim of this study was to identify plants in this system with the potential to reduce methane production and allow us to broaden the plant list outside low-rainfall zones.

Materials and Methods

Plant material

A total of 31 plant samples, including 26 grass samples (from 14 plant species) and 5 legume samples (from 4 plant species) were collected in the period 2003 – 2008 from a variety of locations and in association with ongoing *in vivo* trials in North Queensland (Table 1).

Table 1. List of plant samples analysed

Sample ID	Botanical name	Common name	Origin	Additional info
Grasses				
TV0021	*Astrebla sp.*	Mitchell Grass	Unknown	AGO period 7 (mixed from met crates 1-6)
TV0036	*Astrebla sp.*	Mitchell Grass	Smithfield Ilfracombe	Smithfield Ilfracombe
TV0019	*Bothriochloa insculpta*	Bisset	Brian pastures	AGO period 6 HIGH
TV0020	*Bothriochloa insculpta*	Bisset	Brian pastures	AGO period 6 LOW
TV0005	*Cenchrus ciliaris*	Buffel	Belmont Research Station	Early cut
TV0006	*Cenchrus ciliaris*	Buffel	Belmont Research Station	Medium cut
TV0007	*Cenchrus ciliaris*	Buffel	Belmont Research Station	Old cut
TV0008	*Chloris gayana*	Rhodes	Unknown	PMK 15/04/2003; IVFT CONTROL
TV0009	*Chloris gayana*	Rhodes	Belmont Research Station	Belmont Rhodes 05/12/08
TV0010	*Chloris gayana*	Rhodes	Grame Ika	BCM trial 2003 period 2 mix
TV0011	*Chloris gayana*	Rhodes	Lyle Harder	Used in DSM-CRINA trials 2009
TV0037	*Chloris gayana*	Rhodes Low	Unknown	AGO period 5A offered 16-23

TV0038	*Chloris gayana*	Rhodes High	Unknown	Feb 2009 High Rhodes AGO period 5A offered 16-23 Feb 2009 3g N/kg DM (McCrabb et al 1997). ACIAR #3 Sample
TV0001	*Dichanthium aristatum*	Angelton grass	Unknown	1132
TV0029	*Digitaria decumbens*	Pangola	Belmont Research Station	Pangola grass cut for silage
TV0030	*Digitaria decumbens*	Pangola	Unknown	Low-medium quality Pangola grass hay 2008
TV0002	*Heteropogon contortus*	Speargrass	Belmont Research Station	1.0% N, 74% NDF and 22% leaf. AGO p1 offered
TV0003	*Heteropogon contortus*	Speargrass	Belmont Research Station	0.6%N, 79% NDF and 22% leaf. AGO p1 offered
TV0004	*Lablab purpureus*	Dolichos	Lyle Harder	AGO p1 offered ex Mt Cotton
TV0031	*Lolium sp*	Ryegrass	Mount Cotton	Rye grass Control (71)
TV0025	*Stylosanthes guianensis var intermedia*	Fine stem stylo		
TV0026	*Stylosanthes scabra*	Seca stylo		
TV0027	*Stylosanthes seabrana*	Cattingga Stylo		
TV0023	*Stylosanthes sp.*	Stylo		AGO P9 offered
TV0028	*Triticum sp.*	Wheaten straw	Unknown	cereal hay ex PMK trial 2007 and used in DSM B-carotene trial
Legumes				
TV0022	*Macroptilium bracteatum*	Burgundy Bean	Belmont Research Station	AGO period 8 12-18 June 2009 offered
TV0024	*Centrosema pubescens*	ButterflyPea		DM 60.1% Not from AGO p10

TV0012	*Leucaena leucocephala cv.*	Leucaena	Belmont Research Station	new growth collected across pdk 1 mid Sept 2009 Var. Cunningham
TV0032	*Medicago sativa*	Lucerne	Grame Ika	Lucerne hay used in BCM trial 3&4 2003
TV0033	*Medicago sativa*	Lucerne	Unknown	10/12/2004

In vitro fermentation technique (IVFT)
Samples were analysed *in vitro* for fermentation profiles as described previously (Durmic et al. 2010). *Chloris gayana* (Rhodes grass, TV0008) was used as an internal reference or control for comparison of the grasses and *Medicago sativa* (Lucerne, TV 00032) was used as the standard control for comparison of the legumes.

Results:
Plant samples had different fermentation profiles, with gas production ranging from 44 kPa (*Astrebla sp*. TV0021) to 93 kPa (*Leucaena leucocephala* cv.) and VFA from 81 mmol/L (*Heteropogon contortus* TV0002) to 107 mmol/L (*Leucaena leucocephala* cv.). When compared to the commonly used grass (*Chloris gayana* TV0008), only one grass sample (*Cenchrus ciliaris* TV006) significantly ($P<0.05$) reduced gas and VFA. For the legumes tested, *Centrosema pubescens* and *Macroptilium bracteatum* reduced these fermentation parameters when compared to the control *Medicago sativa* TV 00032 (Table 2). Methane values ranged from 29 mL/g DM (*Astrebla sp*. TV0021) to 60 mL/g (*Stylosanthes seabrana*). Seven grass samples and one legume reduced methane significantly when compared to the respective controls ($P<0.05$), but only two plants, i.e. *Chloris gayana* TV0010 and *Cenchrus ciliaris* TV007 did this without reducing gas and VFA. Acetate to propionate ratio ranged from 2.78 to 3.28, with all grasses having values similar to or higher than the control, but one legume (*Centrosema pubescens*) had significantly reduced values to the control ($P<0.05$). None of the grasses reduced ammonia compared to the control, but all three different species of legumes were lower ($P<0.05$) than lucerne. There was no strong correlation between methane production and gas ($R^2 = 0.51$), VFA ($R^2 = 0.35$) acetate to propionate ratio ($R^2 = 0.02$) or ammonia concentrations ($R^2 = 0.05$). There was some variation in fermentation parameters within the same plant species. For example, variability in methane production was observed between samples collected from three different locations, or different variety ('high' vs 'low') in *Chloris gayana*, or at different stages of growth in *Cenchrus ciliaris*.

Table 2. Fermentation profiles of 31 forages from North Queensland

Botanical name		Gas (kPa)		VFA (mmol/L)		CH_4 (mL/gDM)		A:P		NH_3 (mg/L)	
Grasses											
Dichanthium aristatum	TV0001	59	k	84	k	34	ijk	3.05	ijkl	179	lm
Heteropogon contortus	TV0002	53	k	81	k	34	jkl	3.04	ijklm	216	ijk
Heteropogon	TV0003	51	kl	82	k	30	kl	3.06	hijk	216	ijk

contortus											
Lablab purpureus	TV0004	84	bcde	98	bcdefg	41	efghij	3.13	cdefgh	270	cdef
Cenchrus ciliaris	TV0005	90	ab	103	ab	51	abcd	3.15	bcdef	198	jkl
Cenchrus ciliaris	TV0006	77	defghij	94	efghij	45	cdef	3.10	defghi	195	kl
Cenchrus ciliaris	TV0007	75	fghij	94	fghij	34	ijk	3.10	defghi	194	kl
Chloris gayana	TV0008	77	defgh	96	defghij	46	cdef	2.98	mn	186	lm
Chloris gayana	TV0009	84	bcd	97	defghi	51	abcd	3.08	fghij	249	fgh
Chloris gayana	TV0010	80	cdefg	95	efghij	35	ghijk	2.95	no	179	lm
Chloris gayana	TV0011	77	defgh	97	defgh	40	fghij	2.98	lmn	224	hi
Bothriochloa insculpta	TV0019	80	cdefg	98	bcdefg	46	cdef	3.07	hij	183	lm
Bothriochloa insculpta	TV0020	77	defghij	96	defghij	43	defghi	3.07	ghij	177	lm
Astrebla sp.	TV0021	44	l	91	j	29	kl	3.27	a	222	ij
Stylosanthes sp.	TV0023	79	cdefg	95	efghij	40	fghij	3.15	bcde	270	cdef
Stylosanthes guianensis var intermedia	TV0025	77	defghi	97	defghi	40	fghij	3.17	bcd	279	cde
Stylosanthes scabra	TV0026	78	defg	92	hij	55	abc	3.22	ab	258	defg
Stylosanthes seabrana	TV0027	82	bcdef	97	cdefgh	60	a	3.15	bcde	270	cdef
Triticum sp.	TV0028	69	hij	92	hij	45	defg	2.99	lmn	231	ghi
Digitaria decumbens	TV0029	80	cdefg	94	fghij	59	ab	2.93	no	222	ij
Digitaria decumbens	TV0030	72	ghij	92	ghij	50	bcde	2.96	no	218	ijk
Lolium sp	TV0031	84	bcde	103	abc	44	defgh	3.28	a	284	bc
Astrebla sp.	TV0036	58	k	85	k	35	hijk	3.18	bc	217	ijk
Chloris gayana	TV0037	72	ghij	97	defghi	40	fghij	3.03	ijklm	219	ijk
Chloris gayana	TV0038	76	efghij	99	bcde	54	abc	3.03	jklm	239	ghi
Legumes											
Leucaena leucocephala cv.	TV0012	93	a	107	a	52	abcd	3.10	defghi	254	efg
Macroptilium bracteatum	TV0022	68	j	91	ij	34	ijk	3.09	efghij	275	cdef
Centrosema pubescens	TV0024	69	ij	95	efghij	44	defgh	2.78	p	292	bc
Medicago sativa	TV0032	87	abc	101	bcd	47	cdef	3.00	klmn	323	a
Medicago sativa	TV0033	83	bcdef	98	bcdef	44	defgh	3.10	defghi	308	ab
SEM		1.28		0.69		0.98		0.01		4.40	

Significance – within the same column, values not sharing the same superscript differ ($P<0.05$)

Discussion

The aim of the current study was to investigate diversity in the *in vitro* fermentation profiles of common and novel forage plants in tropical beef production systems. Plants

produced a spectrum of fermentation profiles, from those that had reduced rumen fermentation compared to commonly used forage, to those such as *Lolium sp*., *Cenchrus ciliaris* and *Leucaena leucocephala* cv. that produced more VFA than the control species. The VFA are used as major energy source for the ruminant and providing plants that promote production of these in the rumen may result in production benefits in the animal. The study also revealed plants such as *Chloris gayana* and *Cenchrus ciliaris* with other favourable fermentation profiles (i.e. reduced methane with unaltered microbial fermentation). Variability was also observed within plant species, implying that fermentation profiles, including methanogenic potential in the plant, may be driven by factors such as season, stage of growth or plant location. The current study used opportunistic samples to provide preliminary data on variability in the plans, but further, more structured studies are required to investigate this variability in more detail.

Conclusions:

Amongst common grasses and forages for beef production in north Queensland, it is possible to select for those that support rumen microbial fermentation but have relatively low methane production. Further studies are required to investigate the variability in more detail and the compounds responsible for the antimethanogenic effects.

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Draft paper 2 *- Draft manuscript – targeting Anim Feed Sci Tech*

Variability in the i*n vitro* fermentative traits of tropical legumes and grasses for livestock production systems in Australian tropics

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Abstract
The aim of this project was to examine variability in fermentation traits in selected tropical pastures to identify candidates with favorable *in vitro* profiles. Legumes (*n* = 22) and grasses (*n* = 23) were harvested across three seasons between 2010 and 2011 from the botanical garden at James Cook University, Queensland, Australia. *In vitro* fermentation parameters varied between plant species and across the seasons. Amongst the legume samples collected in the summer, there were 15 samples that had significantly lower (P<0.05) methane production than the control *M. sativa*, but only one amongst these, *Arachis pintoi* Amarilo, did not also inhibit gas or VFA production. The values for methanogenic potential in these low methanogenic plants remained low in winter and spring, but none of these differences were significant compared to the respective positive control. The acetate to propionate ratio was reduced significantly in 20 summer, ten winter and two spring samples, while ammonia was reduced in 11 summer, 11 winter and 17 spring samples. In tropical grasses, all winter and spring samples had significantly lower methane production when compared to the control, but in all of these VFA were also reduced. Amongst these, seven winter samples and 16 spring samples did not reduce gas. The acetate to propionate ration was reduced in all samples, while ammonia was reduced in all winter and spring samples except one. Seasonal variation between fermentation parameters was also observed. It was concluded that different quality and fermentation profiles provide opportunities for productive grazing on these pastures, while offering some methane mitigation options in the context of northern Australia farming production systems.

**Key words:* rumen fermentation, methane, Australian plants, beef grazing

Introduction
Australian agriculture contributes 16% of the national greenhouse gas emissions, with methane contributing 64% of the Australia's greenhouse gases (NGGI, 2006). Apart from methane contribution to greenhouse gas emissions, it is one of the two most significant inefficiencies in ruminant production systems (Johnson and Johnson, 1995). The variation in methane production in the rumen is driven by interacting factors such as

the amount and type of diet consumed, digestibility of dietary carbohydrates, type of carbohydrates, and the activity of rumen microbial species (Kornegay, 1996; Morgavi et al., 2010). There is a possibility to reduce methane formation in the rumen through dietary manipulation. There is evidence that significant variation exists in the methanogenic potential amongst tropical forage plant species in the rumen (Soliva et al., 2008), pasture legumes (Woodward et al., 2002; Woodward et al., 2004) and Australian perennial plant species (Durmic et al. 2010).

Livestock production systems in the Australian tropics are based on year round grazing of native or introduced forage species. However, their effect on ruminal fermentation profiles, in particular the variability in methane production when fermented by the rumen microbes, is unknown. In our preliminary screening of some opportunistic samples from North Queensland tropical forages, we found significant variability in methanogenic potential between different plant species. Amongst grasses, there was as much as a two-fold difference between the lowest (i.e. Bagasse, Mitchell grass, Speargrass; 28-30 mL/g DM) and the highest (Cattingga Stylo; 60mL/g DM) methane producing ones, with some of the low methanogenic ones also supporting normal rumen fermentation (gas and VFA production).
The objectives of the current study was to expand the screening of tropical forage plants, using the samples grown under defined conditions, and to examine variability in these across seasons to identify more candidates that reduce methane while supporting overall rumen fermentation.

Materials and methods
In the current study, plants were grown at James Cook University Townsville, Queensland, Australia and collected in the summer 2010 and winter of 2010 and in spring 2011. A total of 22 legumes and 23 grasses were examined. Samples were collected and processed as described previously (Durmic et al. 2010).

Table 1. List of plant species tested

Botanical name	**Common name**	**Cultivar**	**Type**	**Basic ecology and characteristics**	**Area in Australia**
Legumes					
Arachis paraguariensis? Section Erectoides Krapov.		JCU1	Perennial-introduced	Decumbent growth	Central QLD. Trial at DPI Walkamin. Tablelands
Arachis pintoi Krapov. & W.C. Greg.	Amarillo	Amarillo CIAT 17434?	Stoloniferous perennial-introduced herb	Strong taproot and not restricted by soil texture. Intensive grazing systems	Wet tropics and sub-tropics
Arachis pintoi Krapov. & W.C. Greg.		494	Perennial-introduced		DPI

Calliandra calothyrsus Meisn	Calliandra	No cv available	Perennial-introduced leguminous tree	Metamorphic sandy clays and acid infertile soils. Cut and carry feeding systems. It does not tolerate browsing. Coppicing system may graze the regrowth	Humid/sub-humid tropics
Centrosema molle Mart. Ex Benth.	Centro	Cardillo	Perennial climbing introduced-herb	Strong taproot system. The plant can be used in mixture with grasses or as legume protein bank.	New cultivar no well known. Plant present in sub-humid and humid tropics. Tablelands
Centrosema pascuorum Mart. ex Benth.	Centro, centurion	Cavalcade	Annual-introduced herb	Adapted to a wide range of soils. Mixture with grasses. Hay crop and food pellets. High palatable. Beef production. Product of Australian breeding program between 1976 and 1981	Semi-arid/dry sub-humid Northern Territory. Areas with long dry seasons.
Chamaecrista rotuindifolia	Round-leaf cassia	Wynn cassia	Perennial or self-regenerating annual-introduced herb	Sandy loan soils, tolerant to defoliation and heavy grazing	Drier areas. Townsville, Charters Towers and stylo's niche areas

Clitoria ternatea L.	Blue-pea	Milgarra	Perennial-herbaceous legume	Medicinal plant use as crop for revegetation on coal mines or grazing. Adapted to a wide range of soils, but especially on clay soils no appropriate for leucaena	Central QLD and semi-arid Australia
Desmanthus bicornutus S. Watson		JCU4	Perennial-introduced shrub	Occurs on sandy, rocky and clay soils. Regrows after fire	Semi-arid western QLD and wet-dry North-eastern QLD
Desmanthus leptophillus Kunth	Desmanthus	JCU1	Perennial-introduced shrub	Low-land humid-tropical areas and dry clay soils	Townsville, Charters Towers
Desmanthus virgatus (L.) Willd.	Koa	JCU2	Perennial-introduced shrub with strong basal branching	Present on a range of soil types with emphasis on clay soils in subhumid areas. Tolerant to grazing and dry seasons.	Northern and Western QLD
Desmanthus virgatus (L.) Willd.	Koa	Marc	Perennial-introduced shrub	Adapted to clay soils and less palatable than leucaena and contains condensed tannins	Southern and central QLD
Desmodium heterophyllum (Willd.) DC.	Hetero, guinea clover	Hetero	Introduced and naturalised perennial prostate and stoloniferous legume	Soils with low pH and high Al. Tolerant to heavy grazing, but does not support drought conditions. Moderate level of tannins	Humid North-East Australia
Glycine tabacina (Labill.) Benth.	Glycine pea		Perennial-native legume	Woodland habitat	Townsville, North, East and West

Glyricidium sepicum (Jacq.) Kunth ex Walp.	Gliricidia	CSIRO accession? (No cv available)	Perennial-introduced leguminous tree	Cut and carry, and browse systems. Unidentified alkaloids and tannins. Compatibility with shade tolerant grasses. Drought tolerant	Townsville, wet-dry areas
Leucaena leucocephala (Lam.) De Wit	Leucaena	Tarramba (CSIRO accession?)	Perennial-introduced leguminous tree	Shallow limestone soils. Intolerant to Al or acidic soils. Tolerant to defoliation and up to 7 months dry season. High nutritive value. CT-containing forage	Sub-humid Central and Northern QLD, Townsville, Emerald
Medicago sativa L.	Lucerne	QLD11	Biannual or perennial with deeply tap root system-Introduced	Requires sand to moderate heavy clay soils	
Sesbania sesban (L.) Merr.	Egyptian pea	Mt cotton (UQ)	Shrub-native	Cut and carry or browsing systems. Grows from heavy clays to loose sand soils	Northern territory with reduced used in North QLD
Stylosantes hamata (L.) Taub.	Hamata, Stylo	Verano	Much-branched annual to biannual-introduced legume	Diploids and tetraploids plants adapted to a wide range of pH soils. Tetraploids are appropriate for low rainfall areas	Wide use in areas were Kangaroo, Black spear, Australian bluestem, Golden Beard and Pitted blue grasses are grazed

Stylosantes scabra Vog.	Seca	Seca	Shrubby perennial-introduced legume	Strong deep taproot (~ 4 m) adapted to infertile, acid, friable or hard-setting, sandy-surfaced soils	Associated with Kangaroo, Black spear, Australian bluestem, Golden Beard and Pitted blue grasses (i.e., native) are grazed
Stylosantes seabrana B.L. Maass & 't Mannetje	Caatinga stylo	Caatinga	Perennial-introduced legume (2-4 years)	Clay and clay loam soils, grazing systems and drought tolerant specie	Southern QLD
Vigna laceolata Benth.	Maloga vigna		Native-perennial herb or climber	Black cracking soils	Julia creek Western QLD, Northern Territory

Grasses

Andropogon gayanus
Asdtrebla squarrosa
Astrebla elymoides
Bothriocloa bladhii
Bothriocloa decipiens
Bothriocloa pertusa
Chrsopogon fallax
Dichantium sericeum
Digitaria decumbens
Eulalia fulva
Heteropgon contortus
Heteropgon triteceus
Iaesaeum
Iseilema vaginiflorum
Megathyrsus maximum-Panicum maximum

Penissetum ciliare
Setaria sphacelata
Sorgum plumosum
Themeda triandra
Urochola brizantha-Brachiaria brizantha
Urochola decumbens-Brachiaria decumbens
Urochola humidicola-Brachiaria humidicola
Urochola mosambicensis

Results

The fermentation profiles of tropical legumes varied between the species and across the seasons (Table 2). Amongst the legume samples collected in the summer, there were 15 samples that had significantly lower ($P<0.05$) methane production than the control *M. sativa*, but only one sample, *Arachis pintoi* Amarilo, did not have an associated reduction in total gas or VFA production. The values for methanogenic potential in these low methanogenic plants remained low in winter and spring, but none of these differences were significant compared to the control. Methane concentrations in samples varied from 18 to 48 mL/g DM in summer, from 23 to 39 mL/g DM in winter and from 22 to 44 mL/g DM in spring. The acetate to propionate ratio was lower in 20 summer, ten winter and two spring samples ($P<0.05$), while ammonia was reduced in 11 summer, 11 winter and 17 spring samples.

Tropical grasses also varied in their fermentation profiles. All winter and spring samples had lower methane when compared to the control ($P<0.05$), but in all of these total VFA concentration was also reduced. Amongst the samples with lower methane, seven winter samples and 16 spring did not inhibit gas production. Methane concentrations in samples varied from 38 to 61 mL/g DM in summer, from 20 to 43 in winter and from 21 to 46 mL/g DM in spring. The acetate to propionate ration was reduced in all samples, while ammonia was reduced in all but one of the winter and spring samples.

Table 2. *In vitro* fermentation profiles of 22 tropical legume species across three seasons

	Wet summer 2010					Winter 2010					Spring 2011				
	Gas (kPa)	VFA (mmol/L)	CH4 (mL/g DM)	A:P	NH3 (mg/L)	Gas (kPa)	VFA (mmol/L)	CH4 (mL/g DM)	A:P	NH3 (mg/L)	Gas (kPa)	VFA (mmol/L)	CH4 (mL/g DM)	A:P	NH3 (mg/L)
Medicago sativa	91	107	41	2.8	299	90	74	27	1.8	130	90	68	42	2.0	179
Arachis paraguariensis	99 a	111 a	39	2.5 b	272 b	100	84	23 b	1.9 a	117 b	100 a	70	42	2.1 a	153 b
Arachis pintoi	99 a	114 a	38	2.6 b	211 b	96	93 a	31	2.0 a	156 a	94	70	41	2.2 a	144 b
Arachis pintoi Amarilo	89	114 a	37 b	2.6 b	301	103	89 a	27	2.0 a	84 b	96	70	35 b	2.1 a	131 b
Calliandra calothyrsus	47 b	85 b	18 b	2.5 b	188 b	170 a	62	33	1.9	57 b	62 b	59 b	30 b	2.5 a	129 b
Centrosema molle	72 b	98 b	30 b	2.4 b	316	64	59 b	37 a	1.7 b	113 b	73 b	55 b	34 b	2.0	132 b
Centrosema pascuorum	89	108	39	2.5 b	363 a	97	86	29	1.9 a	167 a	82 b	66	38 b	2.1 a	250 a
Chamaecrista Rotuindifolia	84 b	103 b	35 b	2.5 b	281	86	80	26	1.9 a	127	81 b	67	38 b	2.2 a	214 a
Clitoria ternatea	82 b	106	35 b	2.6 b	327 a	65	68	31	1.6 b	126	85	57 b	37 b	1.7 b	138 b
Desmanthus bicornutus	64 b	96 b	26 b	2.6 b	253 b	72	72	25 b	1.8	107 b	77 b	66	35 b	2.2 a	153 b
Desmanthus leptophillus	62 b	95 b	25 b	2.5 b	229 b	61	72	31	1.9 a	103 b	42 b	53 b	22 b	2.0	63 b

Desmanthus virgatus	58	b	92	b	26	b	2.5	b	230	b	71	68		32		1.9	a	106	b	72	b	57	b	33	b	2.0		70	b
Desmanthus virgatus Marc	75	b	98	b	28	b	2.7	b	225	b	68	70		39	a	1.9		119	b	91		69		41		2.1	a	51	b
Desmodium heterophyllum	77	b	102	b	33	b	2.6	b	303		85	73		32		2.0	a	148	a	79	b	59	b	36	b	2.0		136	b
Glycine tabacina	79	b	104		34	b	2.4	b	331	a	80	71		28		2.0	a	167	a	71	b	59	b	35	b	2.1		156	b
Glyricidium sepicum	83	b	104		35	b	2.7	b	364	a	92	70		36	a	1.8		205	a	91		65		41		2.0		283	a
Leucaena leucocephala	71	b	96	b	26	b	2.5	b	253	b	75	74		28		1.9	a	98	b	68	b	64		31	b	2.3	a	106	b
Sebania sesban	89		101	b	31	b	2.6	b	143	b	73	59	b	30		1.7	b	80	b	85		58	b	38	b	1.8	b	104	b
Stylosantes hamata	94		106		48	a	2.8		369	a	70	66		34		1.9		154	a	97		68		44		2.1	a	201	a
Stylosantes scabra	92		105		38		2.5	b	279	b	60	62		30		1.9	a	160	a	80	b	56	b	33	b	2.1	a	159	b
Stylosantes seabrana	87	b	105		37	b	2.6	b	321	a	89	69		31		2.0	a	142	a	90		65		39	b	2.2	a	165	b
Vigna laceolata	97	a	110	a	38		2.7	b	226	b	92	76		26	b	1.9		70	b	83	b	62		36	b	2.1	a	147	b

Significance: within same column, values significantly (P<0.05) higher (a) or lower (b) than the control species *M. sativa*

Table 3. In vitro fermentation profiles of 23 tropical grass samples across three seasons

	Wet summer 2010					**Winter 2010**					**Spring 2011**				
	Gas (kPa)	VFA (mmol/L	CH4 (mL/g	A:P	NH3 (mg/L)	Gas (kPa)	VFA (mmol/	CH4 (mL/g	A:P	NH3 (mg/L)	Gas (kPa)	VFA (mmol	CH4 (mL/g	A:P	NH3 (mg/L)

			)		DM)							L)		DM)							/L)		DM)											
Chloris gayana	77		96		46	3.0		186		74		91		43	3.0		175		77		96		46		2.2	b	186							
Andropogon gayanus	96	a	132	a	45	2.5	b	233	a	66	b	64	b	25	b	1.9	b	70	b	64	b	63	b	21	b	2.1	b	78	b					
Asdtrebla squarrosa	87	a	119	a	50	2.6	b	295	a	50	b	56	b	21	b	1.9	b	95	b	70		70	b	29	b	2.2	b	178						
*Astrebla elymoides**	94	a	130	a	51	2.5	b	276	a	54	b	60	b	23	b	1.9	b	91	b															
Bothriocloa bladhii*	92	a	141	a	50	2.6	b	258	a	74		64	b	29	b	1.9	b	67	b															
Bothriocloa decipiens	91	a	131	a	40	2.5	b	284	a	63	b	65	b	28	b	2.0	b	71	b	70		67	b	25	b	2.1	b	122	b					
Bothriocloa pertusa	90	a	134	a	43	2.6	b	253	a	65	b	68	b	30	b	2.1	b	78	b	87	a	76	b	35	b	2.0	b	97	b					
Chrsopogon fallax	89	a	130	a	47	2.5	b	290	a	63	b	60	b	23	b	1.9	b	73	b	63	b	60	b	31	b	2.0	b	104	b					
*Dichantium sericeum**	89	a	131	a	48	2.5	b	273	a	72	b	63	b	26	b	1.9	b	66	b															
Digitaria decumbens	102	a	136	a	48	2.5	b	201		96	a	80	b	34	b	2.0	b	50	b	95	a	76	b	29	b	2.1	b	50	b					
Eulalia fulva	89	a	132	a	46	2.6	b	256	a	68	b	64	b	21	b	1.9	b	62	b	58	b	61	b	30	b	2.1	b	81	b					
Heteropgon contortus	94	a	126	a	45	2.5	b	244	a	65	b	63	b	21	b	2.0	b	62	b	72		68	b	24	b	2.0	b	96	b					
Heteropgon triteceus	96	a	131	a	47	2.5	b	256	a	60	b	60	b	21	b	2.0	b	72	b	42	b	60	b	35	b	2.0	b	89	b					
Iaesaeum	97	a	120	a	51	2.6	b	223	a	65	b	70	b	23	b	2.0	b	59	b	67	b	61	b	33	b	2.1	b	66	b					
Iseilema vaginiflorum	81		120	a	50	2.6	b	225	a	60	b	63	b	22	b	2.0	b	60	b	98	a	68	b	34	b	1.8	b	89	b					
Megathyrsus maximum-	98	a	120	a	55	2.5	b	252	a	71	b	64	b	27	b	2.0	b	67	b	93	a	68	b	40	b	2.0	b	103	b					

Panicum maximum																																
Penissetum ciliare	95	a	140	a	47		2.5	b	272	a	63	b	67	b	32	b	2.0	b	71	b	94	a	77	b	31	b	2.1	b	81	b		
Setaria sphacelata	99	a	137	a	56		2.5	b	256	a	78		69	b	25	b	1.9	b	60	b	81		65	b	35	b	2.1	b	71	b		
Sorgum plumosum	95	a	129	a	56	a	2.6	b	280	a	55	b	59	b	20	b	1.9	b	77	b	80		64	b	35	b	1.8	b	82	b		
Themeda triandra	91	a	133	a	48		2.6	b	254	a	71	b	62	b	24	b	1.9	b	63	b	74		66	b	39	b	2.2	b	77	b		
Urochola brizantha-Brachiaria brizantha	109	a	136	a	61	a	2.5	b	194		79		69	b	30	b	1.8	b	25	b	86	a	73	b	33	b	2.1	b	40	b		
Urochola decumbens-Brachiaria decumbens	94	a	130	a	43		2.3	b	162		80		71	b	24	b	1.8	b	17	b	87	a	67	b	34	b	2.1	b	37	b		
Urochola humidicola-Brachiaria humidicola	102	a	136	a	47		2.5	b	233	a	78		72	b	31	b	2.0	b	54	b	94	a	76	b	29	b	2.0	b	55	b		
Urochola mosambicensis	95	a	127	a	38		2.4	b	245	a	92	a	80	b	35	b	1.9	b	83	b	105	a	84	b	29	b	3.0		107	b		

* Spring sample not collected

Significance: within same column, values significantly (P<0.05) higher (a) or lower (b) than the control species *C. gayana*

Discussion

In this study we screened key tropical legumes and grasses for the potential to improve and modulate rumen microbial fermentation for sustainable grazing in North Queensland. Moss et al. (2000) suggested that reducing methane formation in the rumen through dietary manipulation in grazing animal is a possibility if plants that have antimicrobial activities in the rumen can be identified. There is evidence that some tropical plants have antimicrobial activity and that they vary in their methanogenic potential tropical plants (Kamra et al., 2006; Soliva et al., 2008). Our study revealed variability in methanogenic potential amongst tropical legumes and grasses, with some plants producing twice as much methane as others when fermented by the rumen microbes. Where plants caused a reduction in methane, it was often associated with a significant reduction in overall microbial activity, suggesting that the antimicrobial activity in those plants was general, rather than specific against methanogens. However, there were plants, for example the legume *Arachis pintoi* Amarilo (winter sample) or grasses *Urochola mosambicensis* and *Digitaria decumbens* that reduced methane without causing significant reductions in other fermentation parameters. In the current study, the performance of the tropical legume samples were compared to *Medicago sativa* (lucerne), because it is a dominant pasture legume in Australia. Lucerne is often used in the literature as a standard legume species in fermentability studies (Haddi et al., 2003; Tavendale et al., 2005; Li et al., 2010). However, its use in North Queensland may be limited and a comparison with another common legume species such as leucaena might be more relevant for this area. Nevertheless, we have found a range of tropical legume plants with fermentation profiles comparable or better than lucerne. Furthermore, current beef production systems in North Queensland rely on Rhodes grass (*Chloris gayana)*, but our study demonstrated that some other grasses that are suited to the tropics have beneficial properties in the rumen and are worth investigating further. In samples collected in summer, all except one grass species (*Iseilema vaginiflorum*) had rumen fermentation parameters such as gas and VFA production that were significantly better than Rhodes grass.

Our results indicate that there is an opportunity to use some plants for methane mitigation in grazing ruminants in Northern Queensland. However, before the real potential of introducing some of the novel plants into the grazing systems for this purpose can be established, their agronomic properties and nutritive value *in vivo* need to be investigated to determine whether they are comparable to the pasture species that are being used currently. Examining plants *in vitro* is a practical and simple technique to assess the potential bioactivity of feed (Rymer and Givens, 2002; Makkar, 2005), but testing these findings *in vivo* is essential to determine whether the results from the *in vitro* test have functional significance in the rumen of animals.

In conclusion, a variety of Australian tropical forage species demonstrated variability and promising results in the potential to reduce the production of methane in rumen when examined *in vitro*. However, further investigation in plant variability, nutritive composition of plants and possible negative effect of consuming these plants on animal metabolic function, will enable selection of plants that may provide a better manipulation in rumen fermentation. We also intend to pursue the mechanisms behind the antimicrobial and antimethanogenic activity of the plants that resulted in the greatest reduction in methane production when compared to the internal control, particularly the plants that reduced or maintained methane production without worsening other fermentation parameters.

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Draft paper 3 *- Draft manuscript for Advances in Agronomy*

In vitro fermentability and methane production of some potential novel forages in Australia

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Abstract
In vitro ruminal fermentation profiles of some novel forage plants in Australia, including various parts (eg. leaf, bulb) of turnip, broccoli, chicory, plantain, hunter and Winfred, were examined. The extent of fermentation was measured in an *in vitro* batch fermentation system. Overall, turnip Marco maintained normal fermentation (gas and VFA), while reducing methane (bulb and leaf), acetate to propionate ratios and ammonia levels (bulb only). Chicory 1 and plantain also caused a significant reduction in methane and ammonia (plantain only), but they caused a significant reduction in fermentation parameters as well (gas production). The selected forages have fermentability and nutritive values comparable to conventional forages and may have beneficial effects in the rumen. They have the potential to become a valuable supplementary feedstuff for ruminants in Australia.

Introduction
Management strategies for reducing methane emissions in forage-based systems may include selecting plants with the potential to reduce methane when grazed. A range of conventional (Waghorn et al., 2002; Woodward et al., 2004; Tavendale et al., 2005; Ramírez-Restrepoa et al., 2010) and some novel forages (Soliva et al., 2008; Durmic et al., 2010; Ramírez-Restrepoa et al., 2010; Jayanegara et al., 2011) have been reported to have methane-suppressing properties. The plants we have considered in this experiment have been chosen for this purpose, as they may contain plant secondary compounds (PSC) that have bioactive properties and may affect microorganisms in the rumen that influence the amount of methane that is produced. In particular, inclusion of forages containing tannins has been shown to inhibit methane emissions *in vitro* (Hess et al. 2003b, McMahon et al., 1999) and *in vivo* (Woodward et al., 2004). Chicory (*Cichorium intybus L*.) is a tanniferous plant used as a forage for livestock in many parts of the world, providing fodder similar or better to legumes and grass-based pastures (Li and Kemp, 2005). In livestock, it has been shown to reduce methane in grazing sheep (Waghorn et al., 2002), but the effect may be affected by experimental design and level of intake (Sun et al., 2011). Another group of plants considered for this purpose include forage brassicas (radish, turnip, swedes, broccoli, winfred, hunter). Both foliage and roots are readily consumed by the sheep, support rumen function and appear to be utilised efficiently (Kaur et al., 2010), and some forages (eg. turnip) even have a greater animal carrying capacity than

conventional grass pasture (Undersander et al., 2000). Brassicas also contain significant levels of PSC, mostly glucosinolates that have demonstrated bioactivity against crop diseases and gut nematodes (Ayres and Clements, 2002). Broccoli contains the highest amount of sulforaphane amongst vegetables, and this organosulfur compound exhibits antimicrobial properties (Stoewsand, 1995). The secondary compounds also vary within the plant, eg. glucosinolates are generally higher in older forage and in roots compared to leaves (Smith and Griffiths, 1988). The aim of this study was to test selected novel forages for their potential to reduce methane production by rumen microbes *in vitro*.

Materials and methods:

Plant material

The testing included a range of forage species including two types of broccoli (low and high organosulphur), two types of turnip, three samples of chicory, hunter, plantain and two types of winfred. Different parts of the plants were tested in come cases (eg. leaves, bulb) and where this occurred it is stated clearly in the tables and text. The samples were included as the sole substrate in the *in vitro* fermentation (100 mg/10 mL buffered rumen fluid). Two types of clover (*Trifolium repens*) were used as control pasture species (Ramírez-Restrepoa et al., 2010).

In vitro fermentation technique

The material was tested using an *in vitro* batch fermentation assay (Durmic et al., 2010). Briefly, substrate was weighted in Bellco tubes, transferred into an anaerobic chamber where they were filled with 10 mL buffered rumen fluid collected from two fistulated sheep. Tubes were stoppered, crimped and incubated with shaking for 24 h. At the end of the incubation period, gas pressure and methane concentrations in the headspace gas, as well as concentrations of VFA, NH_3 and acetate to propionate ratios were measured as described previously (Durmic et al., 2010).

Results

Forages varied in their fermentability (Table 1). The highest gas pressure was recorded with clover 2 (122 kPa) and the lowest with chicory 2 (91 kPa), with turnip, hunter and winfred 1 (whole plant) producing values comparable to the control, and the others not reducing gas production by more than 25% compared to the clover. Chicory 3 produced the highest (138 mmol/L) and chicory 2 the lowest (98 mmol/L) VFA concentrations and these were significantly different to the control (P<0.05). Significantly higher amounts of methane were produced by Winfred 1 (whole plant) produced higher amounts of methane (57 mL/g DM; P<0.05) than the control, whereas reduced levels of methane were observed with turnip Marco, chicory 1, plantain and winfred 1 leaf fraction (36 - 38 mL/g DM). In these cases, methane production was reduced by up to 25% compared to the control. The acetate to propionate ratio was the highest in two winfred samples (3.6) and the only sample that produced lower values compared to clover was Turnip Marco (bulb) (2.8; P<0.05). This sample also produced the lowest concentrations of ammonia (161 mg/L) but there was also a significant reduction observed with clover 2 and plantain (P<0.05).

There was variability in the effects of different varieties of the same species in this test. Leaf material from Marco variety of turnip produced less methane than the APT variety (P<0.05), the methane produced by chicory 1 was significantly lower than chicory 2, and winfred 1 (whole plant) was more methanogenic than winfred 2. However, broccoli varieties differing in organosulfur content did not perform differently. Variability was also observed between different parts of the same plant – for example in turnip APT, leaf was more methanogenic than its bulb, and leaf or stem material of winfred 1 was less methanogenic than the whole plant.

Table 1. Fermentation profiles of forages incubated *in vitro* with mixed rumen population

Treatment	Gas (kPa)	VFA (mmol/L)	CH_4 (mL/g DM)	A:P	NH_3 (mg/L)
Clover 1 (control)	121	112	48	3.1	235
Clover 2 (3030)	122	104	49	3.0	195 [b]
Chicory 1	95 [b]	114	37 [b]	3.5 [a]	236
Chicory 2	91 [b]	98 [b]	46	3.3 [a]	242
Chicory 3	110 [a]	138 [a]	44	3.5 [a]	211 [b]
Broccoli Booster	121	100	47	3.1	217 [b]
Broccoli Profit	120	101	50	3.0	293 [a]
Turnip APT (bulb)	119	117	44	3.3 [a]	341 [a]
Turnip APT (leaf)	118	122	54	3.4 [a]	322 [a]
Turnip Marco (bulb)	116	121	38 [b]	2.8 [b]	161 [b]
Turnip Marco (leaf)	114	115	37 [b]	3.2 [a]	246 [a]
Hunter	115	120	42	3.0	259 [a]
Plantain	101 [b]	123	36 [b]	3.5 [a]	177 [b]
Winfred 1 (leaf)	108 [b]	125	39	3.6 [a]	377 [a]
Winfred 1 (stem)	103 [b]	113	42	3.3 [a]	337 [a]
Winfred 1 (whole plant)	122	108	57 [a]	3.3 [a]	332 [a]
Winfred 2 (whole plant)	106 [b]	125	42	3.6 [a]	334 [a]
S. E. M.	1.4	1.6	1.0	0.03	8.7

Significance – within the same column, values not sharing same superscript letter are significantly different ($P < 0.05$)

Discussion

We examined selected novel forages for their potential to complement forages that are currently used in grazing systems in Victoria. Our aim was to identify new forages that reduced wasteful processes in the rumen ecosystem, including methane and ammonia production, while stimulating favourable pathways such as propionate production. We have demonstrated that selected forages, including clover 3030, chicory 3, all broccoli and turnip samples, hunter and winfred 1 (whole plant) had fermentability (gas and VFA) profiles comparable to clover. This implies that they have similar effects in the rumen and may therefore produce similar levels of animal production. Some of these plants also reduced methane and acetate to propionate ratios (turnip Marco), and/or reduced ammonia levels (clover 3030, chicory 3, broccoli Booster and turnip Marco). Some plant compounds are known to have a more general inhibitory effect on ruminal bacteria (Benchaar et al., 2008). In our study, both chicory 1 and plantain appeared to have general inhibitory effects on fermentation (gas production), as well as methane (chicory 1) or methane and ammonia (plantain).

Our results have generally been consistent with other reports of the fermentation characteristics of the novel forages we've tested in this experiment. However, we have identified that there is

variability between varieties of the same species and between different parts of the same plant. For example, chicory has been reported to be antimethanogenic (Waghorn et al., 2002), so it is not surprising that one of the chicory samples we tested lowered methane production significantly. What is more interesting is that only one out of three sources of chicory samples had antimethanogenic effects. Similarly, the APT turnip variety was more methanogenic than the Marco variety and the leaf and bulb components of the plants, within the Marco variety, had opposite effects on the acetate to propionate ratio and ammonia production when compared to the control. The same effects on acetate to propionate ratio and ammonia production were not evident in the APT variety. There are clearly differences in the plant secondary compounds in between the varieties that need to be identified to help explain why these differences in effect occur. Brassicas also exhibit antimicrobial properties (Kyung and Lee, 2001), but in our study only some brassica species altered metabolic pathways. In one case, Winfred 1 actually promoted methane production but only when used as a whole plant not just stem. *B. crassifolia* has been shown to decrease methane production *in vitro* (Jayanegara et al., 2009), yet we did not observe any antimethanogenic activity. Moreover, one line of broccoli (Booster) that was developed specifically for increased bioactivity in the gut, did not differ in effect on ruminal microbial fermentation to a standard broccoli line. Having diversity in the effects on fermentation between varieties within a species and between different parts of the same plant is exciting because it provides an opportunity to use these differences to establish the compounds responsible for the favourable effects and the mechanisms of their action.

We have identified novel forages that have fermentation profiles similar to conventional forages and also modulate rumen fermentation in beneficial ways. Our intention is to pursue the varieties of the different species that improved fermentation profiles (reduced methane and ammonia, increased the acetate to propionate ratio) further in a continuous culture system (Rusitec) and confirm these findings *in vivo*. Ultimately, the adoption of some of the novel forages will depend on on their agronomic characteristics, herbage production, nutritive value, how they fit into a grazing system, their persistence under grazing, and overall animal performance. Our results suggest that this should not only be possible, but that there is an opportunity to reduce methane emissions directly through careful choice of species and varieties within species, as well as reduce emissions intensity through improve livestock productivity.

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In vitro screening of plant essential oils, dietary additives, plant extracts and industry by-products for methane mitigation from ruminants in Australia

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Abstract
The objective of this study was to identify feed additives, plant essential oils, plant extracts and industry by-products for their antimethanogenic potential in the rumen using an *in vitro* batch fermentation system. Significant reduction in methane was observed with 21 samples, including eight feed additives (up to 40% reduction), all eight essential oils (up to 75% reduction), two plant extracts (14% reduction) and three industry by-products (up to 37% reduction). Amongst these, only Acidbuff rumen buffer, DHA, Diamond V yeast metabolites, *N. oculata*, Tannin (M027 and M028) and Grape marc crumble did not inhibit gas and VFA production. There were samples with some other interesting effects on rumen fermentation, for example Olive leaf extract caused a three-fold reduction in the acetate to propionate ratio and ammonia when compared to the control. In conclusion, the current study identified some potent ingredients or compounds that can assist in developing novel feed additives to mitigate methane mitigation from the rumen.

Introduction
Feed additives are non-nutritive compounds added to diets to improve animal performance. Antibiotics such as monensin can reduce methane production by ruminal microbes *in vitro* (Wallace et al., 1980; Jalc et al., 1992; McGinn et al., 2004) and *in vivo* (Odongo et al., 2007). However, the use of antibiotics as growth promoters is associated with the risk of developing antibiotic resistance in human pathogens and there is a search for safer and natural alternatives (Barton, 2000). More recently, a range of plant extracts (Broudiscou et al., 2000; Busquet et al., 2006; Bodas et al., 2008), and essential oils (Varga et al., 2004; Calsamiglia et al., 2007; Kumar et al., 2009; Neeta Agarwal et al., 2009) have been reported to have a similar effect *in vitro*, but with only limited *in vivo* effects (Nigel Thomkins, pers. comm.). Other, more successful plant-based additives include tannins (Carulla et al., 2005; Beauchemin et al., 2008). It was demonstrated that feeding tannin extracts to dairy cows reduces methane emissions by up to 29% (Grainger et al., 2008b). Recently, a selection of Australian native shrubs from the genus *Acacia*, *Eremophila*, *Kennedia* and *Rhagodia* have also been identified as having moderating effects on rumen methane production *in vitro* (Durmic et al., 2010). Some marine products such as DHA-edible algae product and a marine microalagae *Nannochloropsis oculata* are also being considered for this purpose. *In vitro*, the addition of DHA-edible algae product inhibited CH_4 as much as 80%, (Fievez et al., 2007), while is *N. oculata* has been reported to have bactericidal activity (Srinivasakumar and Rajashekhar, 2000). Other forms of feed

additives that may assist in methane mitigation include industry by-products. For example, various fruits contain plant secondary compounds that may have anti-methanogenic properties. Grape marc is a by-product of wine making that has been examined as a feed supplement (Hentges et al., 1982). It contains tannins and therefore potential to control rumen microbes in a similar manner to tanniferous forages. Almond hulls are a by-product of the almond industry, containing bioactive triterpenoids (Takeoka et al., 2000). Finally, bentonite, zeolite and diatomaceous earth are naturally occurring silicate minerals that have the ability to absorb, hold, release, and exchange different chemicals, nutrients and toxins (Trckova et al., 2004), with bentonite demonstrating some moderating effect on rumen fermentation and methane production *in vitro* (Váradyová et al., 2003).
The aim of this study was to test selected feed additives, essential oils, plant extracts, industry by-products and naturally occurring silicate minerals for their potential to reduce methane in the rumen.

Materials and methods:
A range feed additives (n = 12), plant essential oils (n = 8), plant ethanolic extracts (n= 10), industry by-products (n = 11) and naturally occurring silicate minerals (n=5) were tested for their effect on rumen fermentation *in vitro*. The treatments and inclusion levels are listed in Table 1. As these dietary manipulations target intensive systems based on concentrate diets, we used two concentrate-based diets (Milne standard pellets, Milne Feed, Western Australia and Ellinbank pellet, DPI Victoria) as substrates for testing the additives. They were included at levels that correspond to the recommended levels of intake reported in the literature.

In vitro fermentation technique
The material was tested using an *in vitro* batch fermentation assay (Durmic et al., 2010). Briefly, substrate was weighted in Bellco tubes, transferred into and anaerobic chamber where they were filled with 10 mL buffered rumen fluid collected from two fistulated sheep. All additives were separately dissolved in 1 mL rumen fluid to make a stock solution, and then 100 µL of this mixture was added to the treatment tube containing substrate and buffered rumen fluid. Tubes were stoppered, crimped and incubated with shaking for 24 h. At the end of the incubation period, gas pressure and methane concentrations in the headspace gas, as well as concentrations of volatile fatty acids (VFA), ammonia and acetate to propionate ratios were measured.

Results
Feed additives
Inclusion of nine feed additives with control diet resulted in significant reduction in methane production when compared to the control ($P<0.05$; Table 1). The additives caused reductions of 30% - 40% (26 – 61 mL/g DM) in methane production when compared to the control diet without the additive (46 mL/g DM), with the lowest methane values recorded with Tannins (26 - 38 mL/g DM), *N. oculata* (27 mL/g DM), and Agolin (32 mL/g DM). Tannin M020 and Agolin also caused some reduction in total gas production. While DHA reduced methane (33 mL/g), oxidised DHA (50 mL/g DM) did not. Olive leaf extract did not reduce methane, but reduced the acetate to propionate ratio significantly (1.1) and ammonia (37 mg/L) compared to the control (3.0 and 288 mg/L, respectively).

Essential oils
All essential oils caused significant reduction ($P<0.05$) in methane, ranging from 11 mL/g DM to 31 mL/g DM and with level of reduction in some cases up to 75% of the control without essential oils added (*Melaleuca ericifolia* and *M. teretifolia*). However, all of them also reduced gas and

VFA production (P<0.05). All except *Santalum spicatum* had increased acetate to propionate ratios and reduced ammonia levels.

Plant extracts
The addition of two plant extracts, i.e. *E. glabra* and *K. prorepens* resulted in significant (P<0.05) decrease in methane (38 mL/g DM each) compared to the control (44 mL/g DM). These plant extracts, as well as two others caused significant (P<0.05) reduction in gas, but not in VFA. *E. glabra* along with another one significantly reduced acetate : propionate, and along with another five also reduced ammonia.

Industry by-products
Amongst industry by-products, only the inclusion of Almond hulls, Grapemarc crumble or pellet produced methane values (34 , 29 and 30 mL/g DM respectively) that were significantly ($P < 0.05$) lower than the control diet, causing between 25% - 37% reduction. Two of these had some reduction in gas, but they all supported VFA formation comparable to the control. These three samples also significantly increased acetate : propionate ratio, while Almond hulls, along with Bentonite also reduced ammonia levels.

Table 1. Fermentation profiles of different dietary treatments incubated *in vitro* with mixed rumen population

Treatment	LVL*	Source	Category	Gas (kPa)		VFA (mmol/L)		CH_4 (mL/g DM)		A:P		NH_3 (mg/L)	
Experiment 1													
Milne lamb pellet	sole substrate	Milne Feeds, WA	Control	103		86		46		2.3		288	
Agolin	0.1 mg	Feedworks, Vic	Feed additive	91	b	85		32	b	2.4		308	a
CRINA	10 mg	Feedworks, Vic	Feed additive	106		79		46		2.2		312	a
DHA	164 mg	DPI Vic	Feed additive	99		89		33	b	2.2		330	a
DHA oxidised	164 mg	DPI Vic	Feed additive	108		86		50		2.2		343	a
Diamond V yeast metabolites	2.2 mg	Feedworks, Vic	Feed additive	96		88		35	b	2.4		307	a
Monensin	0.5 mg	Feedworks, Vic	Feed additive	103		86		44		2.2		315	a
N. oculata	164 mg	DPI Vic	Feed additive	95		81		27	b	2.2		384	a
Tannin (M020)	20 mg	DPI Vic	Feed additive	86	b	86		26	b	2.4	b	270	
Tannin (M027)	20 mg	DPI Vic	Feed additive	91		90		38	b	2.5		387	a
Tannin (M028)	20 mg	DPI Vic	Feed additive	90		89		37	b	2.6	a	370	a
Agonis fragrans	250 uL	Paperbark Co., WA	Essential oil	69	b	54	b	26	b	4.6	a	259	b
Eucalyptus plenissima	250 uL	Paperbark Co., WA	Essential oil	73	b	56	b	30	b	5.6	a	237	b
Eucalyptus staigeriana	250 uL	Paperbark Co., WA	Essential oil	54	b	41	b	13	b	3.9	a	219	b
Leptospermum pettersoni	250 uL	Paperbark Co., WA	Essential oil	53	b	44	b	14	b	4.3	a	202	b
Melaleuca alternifolia	250 uL	Paperbark Co., WA	Essential oil	78	b	62	b	31	b	5.7	a	251	b
Melaleuca ericifolia	250 uL	Paperbark Co., WA	Essential oil	58	b	51	b	11	b	4.1	a	246	b
Melaleuca teretifolia	250 uL	Paperbark Co., WA	Essential oil	37	b	37	b	11	b	3.9	a	223	b
Santalum spicatum	250 uL	Paperbark Co., WA	Essential oil	74	b	80	b	25	b	2.0	b	283	
Acidbuff	8 mg	Feedworks,	Feed	95		86		33	b	2.3		307	a

rumen buffer		Vic	additive											
Almond hulls	500 mg	DPI Vic	Industry by-product	90	b	91		34	b	2.5	a	203	b
Grapemark crumble	sole substrate	DPI Vic	Industry by-product	97		81		29	b	2.6	a	293	
Grapemark pellets	sole substrate	DPI Vic	Industry by-product	87	b	79		30	b	2.6	a	297	
Zeolite VIC	40 mg	DPI Vic	Industry by-product	104		77		49		2.3		287	
Experiment 2														
Milne lamb pellet + EtOH	sole substrate + 1 mL EtOH	Milne Feeds, WA	Control	104		101		44		2.7		268	
Acacia saligna	1 mL	FFI Enrich	Plant extract	85	b	92		50		2.5	b	143	b
Atriplex nummularia	1 mL	FFI Enrich	Plant extract	107		109		54	a	2.9		266	
Chameacytisis palmensis	1 mL	FFI Enrich	Plant extract	111		114	a	50		3.0	a	248	b
Cullen australasicum	1 mL	FFI Enrich	Plant extract	95	b	98		48		2.5	b	268	
Enchylaena tomentosa	1 mL	FFI Enrich	Plant extract	102		99		57	a	2.7		262	
Eremophila glabra	1 mL	FFI Enrich	Plant extract	91	b	98		38	b	2.2	b	229	b
Eremophila longifolia	1 mL	FFI Enrich	Plant extract	115	a	102		47		2.7		193	b
Kennedia prorepens	1 mL	FFI Enrich	Plant extract	96	b	106		38	b	2.7		151	b
Maireana brevifolia	1 mL	FFI Enrich	Plant extract	106		107		53	a	2.9	a	299	a
Rhagodia preissii	1 mL	FFI Enrich	Plant extract	104		92		54	a	2.7		225	b
Experiment 3														
Ellenbank pellet	sole substrate	DPI Vic	Control	85		83		47		3.0		136	
Corn silk	sole substrate	Waneroo markets, WA	Industry by-product	87		126	a	47		2.9		147	a
Diatomaceous Earth BB	20 mg	Bed Bug Specialist,	Industry by-	92		87		48		3.0		143	

		WA	product											
Diatomaceous Earth BB	50 mg	Bed Bug Specialist, WA	Industry by-product	82		86		47		3.0		137		
Diatomaceous Earth GD	20 mg	Gardeners Direct, WA	Industry by-product	89		88		56	a	3.0		140		
Diatomaceous Earth GD	50 mg	Gardeners Direct, WA	Industry by-product	88		84		47		2.9		135		
Bentonite	50	Gardeners Direct, WA	Industry by-product	79		83		45		3.0		124		b
Zeolite WA	50 mg	Gardeners Direct, WA	Industry by-product	85		84		51		3.0		136		
Olive leaf extract	1 mL	EnvirOlea, QLD	Feed additive	136	a	92		61	a	1.1	a	37		b

Significance – within the same column and in same experiment, values not sharing same superscript letter are significantly different ($P < 0.05$)
* LVL - Level of inclusion of additive per gram of substrate

Discussion

In this study we examined a range of products that can be added to ruminant diets that were considered to have the potential to reduce some of the 'wasteful' processes in the rumen ecosystem. The supplementation of selected commercial feed additives to a concentrate-based diet resulted in significant reduction in methane *in vitro*. Plant-based additives such as tannins and marine extracts were also very effective, causing approximately a two-fold reduction in methane, without severely disrupting normal gas production. Commercial feed additives such as CRINA and monensin, at the levels applied here, were ineffective at reducing methane. Furthermore, DHA, when exposed to air over several days, became ineffective and instead produced 34% more methane than the unaltered form of DHA. The latter implies instability of some compounds and highlights the necessity to further define conditions under which these products may be active.

Essential oils from Australian plants were highly effective in reducing methane, with the level of reduction in the case of *Melaluca teretifolia* being 75% of the control. However, the effect was associated with a significant reduction in fermentation. It has been demonstrated that while EO are active at high doses, they can also inhibit VFA production (Kumar et al., 2009), which could compromise energy supply to the ruminant. There are some cases where the addition of EO have been reported to increase VFA production (Castillejos et al., 2006; Benchaar et al., 2007), whereas others showed no effect (Newbold et al., 2004). This implies that the ultimate effect of EO is probably dependent on other conditions, for example the dose and the base diet (Benchaar et al., 2001). One interesting finding is that olive leaf extract promoted extensive propionate production and reduced ammonia. Propionate is a valuable energy source for the animal, while ammonia is a waste of nitrogen and energy. This plant extract has never been examined for use in ruminant livestock industries, but the findings from this study suggests it warrants further investigation into the secondary compounds that exist in olive leaves (and extract) its possible applications in animal diets. There is likely to be tannins associated with the

olive leaves and their extract which may explain the reduction in ammonia production, but it is unclear why propionate is being promoted so significantly.

In our study, only two plants extracts, *E. glabra* and *K. prorepens,* were capable of reducing methane production when included as ethanolic extracts. These two plants were identified previously as the most potent antimethanogenic plants among 120 Australian native shrubs tested (Durmic et al., 2010). In the previous study, when included as a whole plant, they also inhibited fermentation, but in the current study, when included as extracts, the effect was not associated with inhibition of fermentation. It has been suggested that effects of plant extracts on methane production appears only when they are included at very high levels (Śliwiński et al., 2002). Furthermore, plant extracts, even when selected based on their known bioactivity, often have limited effect on rumen methanogenesis. For example, Bodas et al. (2008) found only 15 antimethanogenic plant extracts amongst 450 tested. Our results support this and suggest that plants selected based on their known bioactivity when included as a whole plant, need to be extracted with a number of organic solvents (and water) to extract the compounds responsible for the antimethanogenic effects observed.

Industry by-products such as almond hulls and grape marc also demonstrated some potential to alter methane production *in vitro*. This is the first report on their activity in the rumen. Other industrial by-products apparently were not effective, which differs from the results reported by Váradyová et al. (2003). However, we tested the industrial by-products in lower amounts and in a system that may have been unfavourable for these mineral-based additives because some were less soluble in rumen fluid than others, which may have led to less reliable results. We are interested to test some of these further in a modified system that overcomes some of these limitations.

We have tested a range of dietary additives in an in vitro fermentation system to identify those that have a positive influence on rumen fermentation, in particular the ability to reduce methane production. To our knowledge, this is the first time that some of these additives have been tested for their effects in a livestock system and the effects of one in particular, olive leaf extract, warrants closer investigation. The results from testing tannins and marine extracts were also encouraging and need to be examined further. We have confirmed that the effects of dietary additives on ruminal fermentation may differ depending on the type of the additive, the form and the dose (Benchaar et al., 2001; Busquet et al., 2006), the nature of the diet (Wallace, 2004), as well as overall ruminal environment (Cardozo et al., 2005). The variability in response among products tested here provides the opportunities to select the highly potential and efficient feed supplements. We need to pursue these candidates in a continuous flow system (Rusitec) to clearly define the conditions (diet, dose, form) when they are the most active and confirm these findings *in vivo* .

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Section 2. Characterise the variability in anti-methanogenic activity in plant materials as influenced by plant collection site, phenological stage and season. (1 draft paper)

Draft manuscript – Anim. Prod. Sci./Anim. Feed Sci. Tech/Agronomy Journal

In vitro methanogenic potential of Australian native perennial shrubs species in the rumen is affected by plant location, season, phenology, grazing status and accession.

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Introduction

Agronomic and nutritional aspects of perennial shrub plants are being investigated in Australia to target low-medium rainfall areas as they may provide out-of-season fodder (Revell et al., 2006; Kumara Mahipala et al., 2009b), complemented with some other extra-nutritional effects (Kotze et al., 2009; Bennell et al., 2010). In ruminants, plant material is fermented by the gut microbes to provide energy for the animal, but during this process, methane is produced as a waste product. Plants produce different fermentation profiles in the rumen, depending on their chemical composition, i.e. nutritive value and the presence of plant secondary compounds (PSC). Chemical composition and fermentability of the plants may be influenced by many factors, such as plant species and type (Sivakumaran et al., 2004; Hayes et al., 2009), plant age or phenology (Smith and Griffiths, 1988; Haddi et al., 2003; Cirak et al., 2007), grazing (Papachristou et al., 2003; Rogosic et al., 2008), or growth conditions such as season (Dement and Mooney, 1974; McDonald and Ternouth, 1979; Smith and Griffiths, 1988) and soil type (Tefera et al., 2008).

In our previous study, we observed a wide spectrum of fermentation profiles amongst different species of Australian native shrubs exists (Durmic et al., 2010). We also observed variability in fermentation amongst opportunistic samples of the same species collected from different locations and at different ages. Our aim in this experiment was to conduct a more systematic approach to studing the variability in the *in vitro* fermentation characteristics of selected Australian native shrubs as influenced by location, season, phenology, grazing or plant accession.

Materials and methods

A total of 11 plant species of perennial forage shrubs were tested (Table 1). Ten plant species were selected to test the effect of plant location on variability in fermentation, and for this

purpose plants were grown and collected at three different experimental locations in Australia – in 2008 at Monarto in South Australia (SA), and in 2010 at Condobolin in New South Wales (NSW) and Merredin in Western Australia (WA). Five different accessions of *Cullen australasicum* were grown at Waite SA, and collected over four seasons (2008/2009) to test seasonal variability. Nine plant species grown at Monarto were examined at different stages of growth (vegetative, reproductive flower, reproductive fruit) during 2009 - 2010. Four species were examined for variability due to grazing of the plant and for this purpose, plants were grown at Monarto and tested as pregrazing (2009) and over two grazing seasons (2009 and 2010). During each grazing season, samples were collected separately from plants that were grazed, and the ones that were excluded from grazing, ie. left ungrazed (grazing control). Finally, 20 accessions of *Cullen australasicum* (Waite, SA) and seven accessions of *Eremophila glabra* (Monarto, SA) were grown in 2009 and tested for variability between accessions. Edible mass (i.e. leaf material and stems up to 5 cm long) of each plant specimen was collected, processed and tested in an *in vitro* batch fermentation system as described previously (Durmic et al., 2010).

Table 1. List of plants and potential source of variation tested

Botanical name	Accession	Potential source of variability tested				
		Location	Season	Phenology	Grazing	Accession
Acacia saligna	SA 44520	X		X		
Atriplex nummularia	cv. Eyres Green	X		X	X	
Chameacytisis palmensis	SA 22699	X		X		
Cullen australasicum	SA 42690	X	X			X
Cullen australasicum	SA 41020		X			X
Cullen australasicum	SA 44286		X			X
Cullen australasicum	SA 44775		X			
Cullen australasicum	SA 45562		X			X
Cullen australasicum	SA 4966		X			X
Cullen australasicum	SA 41272					X
Cullen australasicum	SA 42723					X
Cullen australasicum	SA 42741					X
Cullen australasicum	SA 42745					X
Cullen australasicum	SA 42751					X
Cullen	SA 42766					X

australasicum					
Cullen australasicum	SA 42772				X
Cullen australasicum	SA 42808				X
Cullen australasicum	SA 42825				X
Cullen australasicum	SA 42965				X
Cullen australasicum	SA 44239				X
Cullen australasicum	SA 44383				X
Cullen australasicum	SA 45574				X
Cullen australasicum	SA 4685				X
Enchylaena tomentosa	SA 41119	X	X		
Enchylaena tomentosa	SA 44099			X	
Eremophila glabra	SA 44440	X	X		X
Eremophila glabra	F01				X
Eremophila glabra	SA 44541				X
Eremophila glabra	SA 45577				X
Eremophila glabra	SA 45588				X
Eremophila glabra	SA 45599				X
Eremophila glabra	SA 45608				X
Eremophila glabra	UWA PH				X
Eremophila glabra	CALM				X
Eremophila longifolia	SA 45610	X	X		
Kennedia prorepens	SA 41349	X	X		
Maireana brevifolia	SA 42869	X	X		
Rhagodia parabolica	SA 44469			X	
Rhagodia preissii	SA 45214	X	X	X	

Results

Plant location

Fermentability of plants was influenced by location where plants were grown (Figure 1). Total gas production was lower in samples from Monarto (54 kPa) than the other two sites (74 kPa and 75 kPa; $P < 0.05$), while VFA did not differ. The methanogenic potential of the plants also varied significantly ($P < 0.05$), with the lowest average values recorded from Monarto samples

(16 mL/g DM), followed by Condobolin (47 mL/g DM) and Merredin (54 mL/g DM). There was significant variation in fermentability present in the individual species ($P < 0.05$; individual data not shown). For example, *A. saligna* grown at Monarto produced nine times less methane than the same plant grown at Merredin. Differences were also observed in *A. nummularia,* which produced nearly five and six times more methane when grown at Condobolin and Merredin, compared to Monarto. Individual plant cumulative ranking in methanogenic potential varied across the location (Figure 2).

Figure 1. Fermentation profiles of ten focus species collected from three different locations (site average ± SEM)

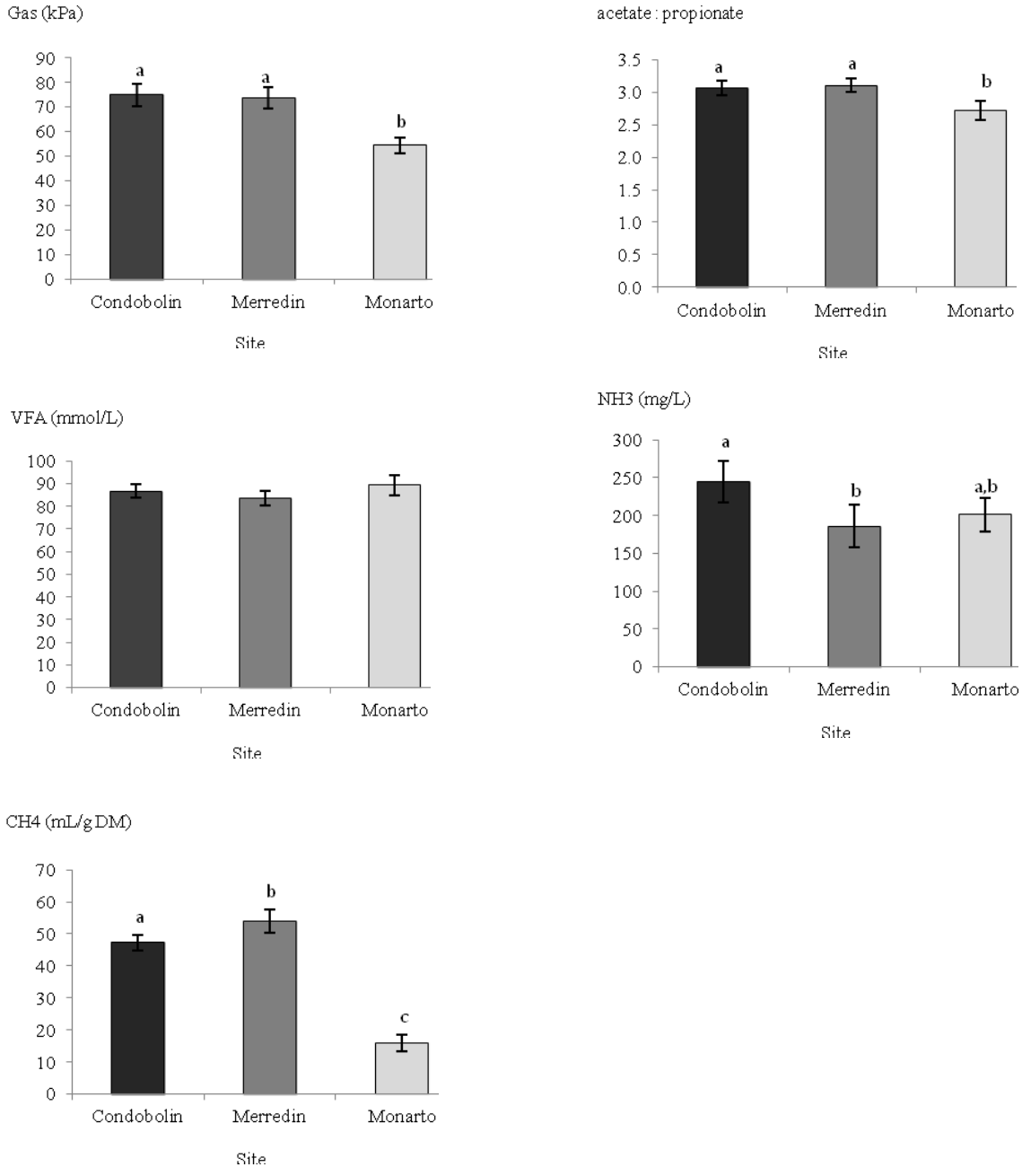
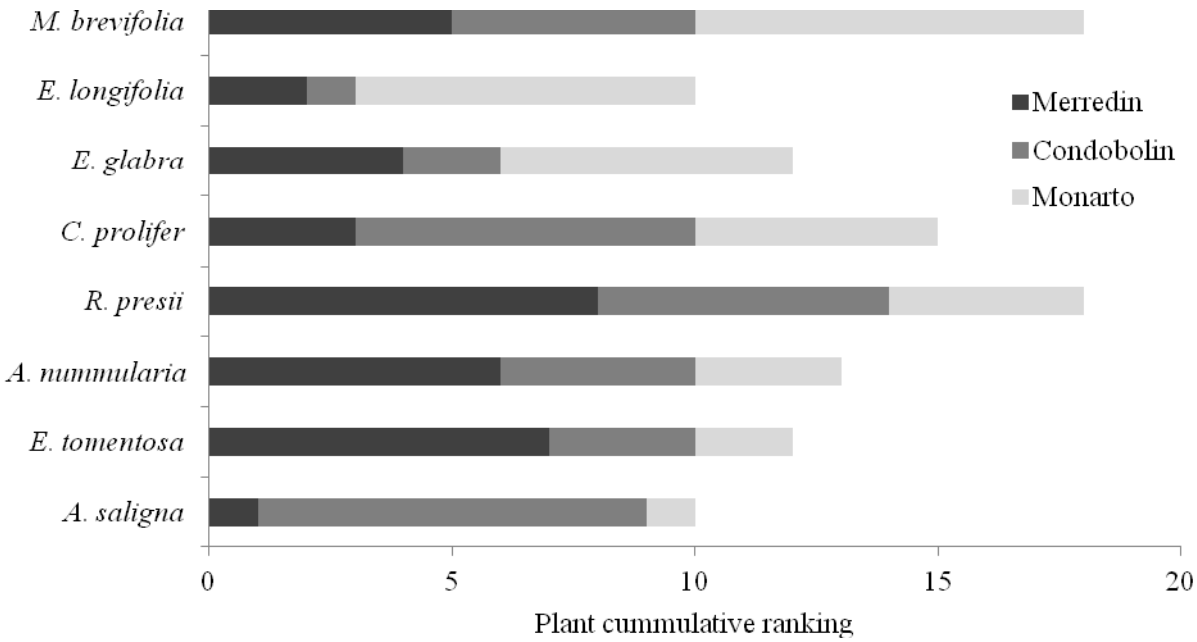


Figure 2. Variability in plant cumulative ranking according to methanogenic potential amongst eight species across three locations



Season

All fermentation parameters were affected by season ($P < 0.05$), but only in selected accessions (Table 2). Gas production varied between autumn (77 – 108 kPa), winter (97 – 109 kPa), spring 104 – 113 kPa) and summer (109 – 115 kPa), but the differences were not significant except for SA 41020 (Table 2). In some accessions, methane production was low in autumn and high in summer (SA 42690 and SA 4966), while in the others it was the opposite (SA 44286 and SA 45562). The differences ranged 15 – 40%, with accession SA 41020 produced as much as 40% more methane in winter than in autumn. All winter samples had the lowest VFA (99 – 101 mmol/L), followed by summer (102 – 111 mmol/L), spring (108 – 115 mmol/L) and autumn (118 -125 mmol/L). Acetate : propionate also differed between autumn (3.0), winter (2.3 – 2.6), spring (2.2 – 2.3) and summer (2.0 – 2.2) and ammonia autumn (401 – 491 mg/L), winter (414 – 465 mg/L), spring (324 – 408 m/L) and summer (311 – 398 mg/L). However, there were no clear trends amongst these parameters and the season individual cumulative plant ranking in methanogenic potential was consistent in some and varied in others across (Figure 3).

Table 2. Fermentation profiles (mean ± S.E.M.) of five *Cullen australasicum* accessions collected at Waite, SA across different seasons in 2009

Accession	Fermentation parameter			
	Gas (kPa)			
	Autumn	Winter	Spring	Summer

SA 41020	77	a	106	b	109	b	111	b
SA 42690	106		103		106		111	
SA 44286	97		97		104		101	
SA 45562	105		107		108		109	
SA 4966	108		109		113		115	
S.E.M.	5.77		1.21		6.57		6.43	

Methane (mL/g DM)

	Autumn		Winter		Spring		Summer	
SA 41020	40	a	66	b	46	a	49	a
SA 42690	35	a	42	ab	49	ab	51	b
SA 44286	53		52		52		45	
SA 45562	75	a	57	b	54	b	54	b
SA 4966	45		53		50		54	
S.E.M.	4.33		3.61		3.23		3.08	

VFA (mmol/L)

	Autumn		Winter		Spring		Summer	
SA 41020	125	a	99	c	117	b	107	c
SA 42690	118	a	101	c	108	bc	110	b
SA 44286	125	a	99	c	115	b	102	c
SA 45562	123	a	97	c	115	b	103	c
SA 4966	124	a	101	c	111	b	111	b
S.E.M.	1.44		0.84		6.90		6.33	

Acetate : Propionate

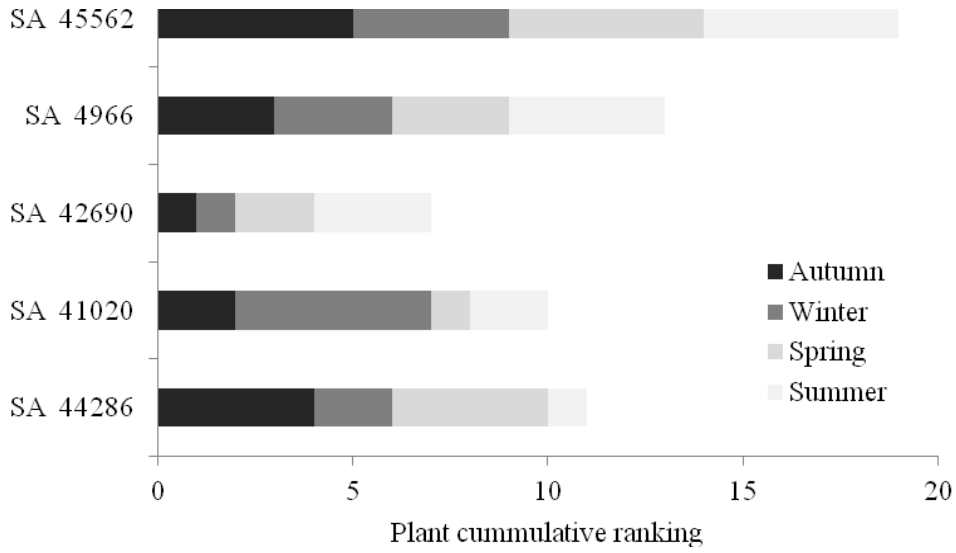
	Autumn		Winter		Spring		Summer	
SA 41020	3.0	a	2.5	d	2.3	b	2.2	c
SA 42690	3.0	a	2.6	c	2.2	b	2.2	b
SA 44286	3.0	a	2.3	d	2.2	b	2.0	c
SA 45562	2.9	a	2.5	c	2.2	b	2.2	b
SA 4966	3.0	a	2.6	d	2.3	b	2.2	c
S.E.M.	0.02		0.03		0.14		0.13	

NH3 (mg/L)

	Autumn		Winter		Spring		Summer	
SA 41020	423	a	414	a	368	b	334	c
SA 42690	491	a	457	c	388	b	398	b
SA 44286	416	a	465	c	408	a	362	b
SA 45562	412	a	423	a	324	b	311	b
SA 4966	401	a	438	d	362	b	323	c
S.E.M.	8.81		6.13		24.61		21.10	

Significance: within each row, values not sharing the same superscript differ (P< 0.05)

Figure 3. Variability in plant cumulative ranking according to methanogenic potential amongst eight six accessions of *C. australasicum* across four different seasons



Phenology

Plant fermentation profiles varied little between phenology stages (Table 3). Gas production did not vary, while all other parameters were the highest ($P < 0.05$) in the flower/fruit stage. Methane production was the lowest in the fruit stage, but the differences between high and low stage, although significant, were very small (i.e. only 8 mL/g DM).

Table 3. Fermentation profiles (group mean ± S.E.M.) of plants across different phenology stages

Phenology stage	Fermentation parameter								
	Gas (kPa)	VFA (mmol/L)		CH4 (mL/g DM)		A:P		NH3 (mg/L)	
Vegetative	63	62	c	26	ab	2.96	b	260	b
Flower	64	70	ab	23	b	2.85	b	235	b
Flower/Fruit	66	74	a	29	a	3.21	a	320	a
Fruit	58	62	bc	21	b	2.96	ab	236	b

Significance: within each column, values not sharing the same superscript differ ($P< 0.05$)

Grazing

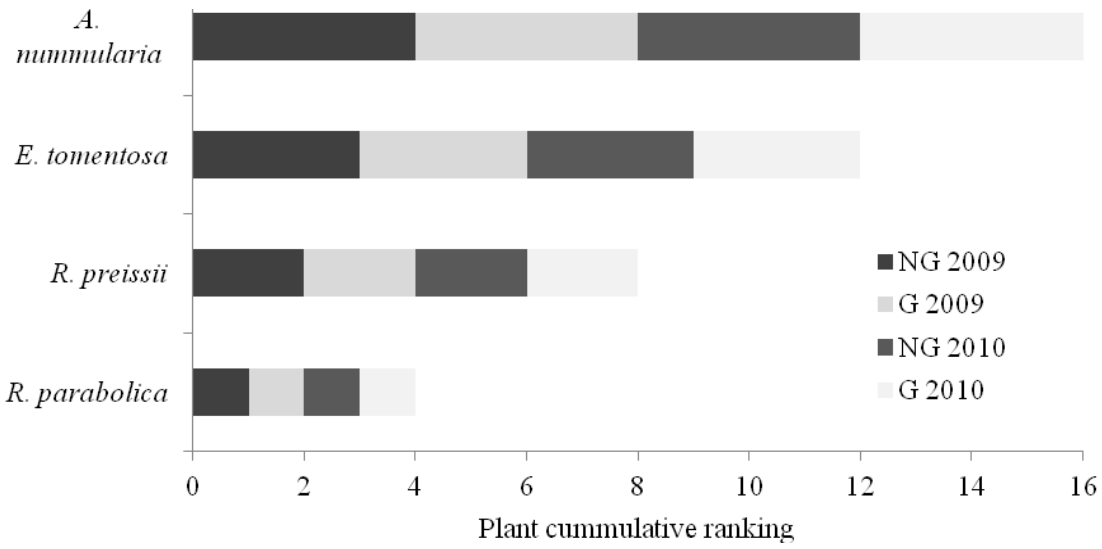
In the first year of grazing (2009), there were no significant differences in gas and VFA profiles, except for the two *Rhagodia* species where grazed plants were more fermentable (higher gas and VFA values) compared to ungrazed (Table 4). Also, there were no significant differences in methanogenic potential of the ungrazed vs grazed plants, except for *A. nummularia* (54 vs 41 mL/g DM). However, ammonia concentrations varied with grazing, with increased values with grazing observed with *E. tomentosa* and *A. nummularia*, and decreased with *R. preissii* and *R. parabolica*.
In the second year of grazing (2010), grazed plants were in general more fermentable (higher gas and VFA values) compared to ungrazed controls (Table 4), but there only few significant ($P < 0.05$) differences in fermentation profiles. In *R. presii*, grazed plant produced significantly ($P<0.05$) more gas (68 kPa) than the control (61 kPa), while grazed *A. nummularia* produced less methane (23 mL/g DM) than the ungrazed control (31 mL/g DM). There were no differences between acetate : propionate, but fermentation of *E. tomentosa* and *R. parabolica* resulted in significantly more ammonia ($P<0.05$) in grazed (311 mg/L and 265 mg/L) than the ungrazed controls (276 mg/L and 172 mg/L), while in *A. nummularia* the opposite occurred (222 mg/L in grazed and 284 mg/L in ungrazed). Individual cumulative ranking of the plants did not vary across the season or between the years (Figure 4).

Table 4. Fermentation profiles of focus plant species as affected by grazing in 2009 and 2010

Treatment	Gas (kPa)			VFA (mmol/L)			CH4 (mL/gDM)			A:P			NH3 (mg/L)		
	NG	G		NG	G		NG	G		NG	G		NG	G	
Grazing in 2009															
E. tomentosa	84	86		113	112		40	39		2.47	2.50		177	223	*
A. nummularia	97	100		121	125		54	41	*	1.71	2.65	*	234	318	*
R. preissii	83	96	*	113	123	*	33	43		2.61	2.72	*	174	145	*
R. parabolica	74	87	*	123	121		32	33		2.36	2.39		217	196	*
S. E. M.															
Grazing in 2010															
E. tomentosa	57	62		67	71		27	23		3.09	3.09		276	311	*
A. nummularia	66	70		68	69		31	32	*	3.16	3.07		284	222	*
R. preissii	61	68	*	69	73		22	24		3.04	3.17		241	222	
R. parabolica[a]	56	57		60	65		21	19		2.81	2.89		172	265	*
S. E. M.															

NG – non-grazed; G – grazed
[a] grazed but not eaten at all (Emms, pers. comm.)
*- within a row and a fermentation parameter, value significantly different ($P<0.05$) between non-grazed and grazed plant

Figure 4. Variability in plant cumulative ranking according to methanogenic potential amongst four species in response to grazing



Accessions

E. glabra

Fermentability parameters varied significantly ($P < 0.05$) in different accessions of *E. glabra* (Table 5). Gas and methane were the lowest in UWA PH (37 kPa and 14 mL/g DM) and highest in SA 45588 (93 kPa and 47 mL/g DM). The accession with the lowest VFA and acetate : propionate was F01 (62 mmol/L) and the highest values were in SA 44541 (101 mmol/L and 2.51). Accession SA 44440 produced the lowest ammonia levels (57 mmol/L) and SA 45588 had the highest levels (174 mmol/L).

Table 5. Fermentation profiles of various accessions of *E. glabra* tested

Accession	Gas (kPa)	CH4 (mL/g DM)	VFA (mmol/L)	A:P	NH3 (mg/L)
SA 44440	47 ^{g}	22 ^{ef}	72 ^{de}	1.64 ^{h}	57 ^{h}
SA 45588	102 ^{a}	47 ^{a}	86 ^{b}	2.44 ^{c}	174 ^{b}
F01	39 ^{i}	16 ^{g}	62 ^{f}	1.64 ^{h}	76 ^{g}
SA 45577	62 ^{f}	21 ^{ef}	88 ^{b}	1.83 ^{g}	135 ^{c}
SA 44541	95 ^{b}	38 ^{c}	101 ^{a}	2.51 ^{b}	128 ^{cd}
SA 45608	63 ^{f}	19 ^{f}	83 ^{bc}	1.81 ^{g}	71 ^{g}
SA 45599	66 ^{e}	23 ^{e}	84 ^{bc}	2.07 ^{e}	81 ^{g}
SA 45599	70 ^{d}	27 ^{d}	82 ^{bc}	2.23 ^{d}	98 ^{f}
UWA PH	37 ^{i}	14 ^{g}	71 ^{de}	2.01 ^{ef}	129 ^{cd}
CALM	43 ^{h}	15 ^{g}	77 ^{cd}	1.96 ^{f}	114 ^{e}
S. E. M.	0.7	0.9	2.6	0.02	4.1

Significance: within the same column, values not sharing the same superscript differ ($P < 0.05$)

C. australasicum

Overall, the accessions of *C. australasicum* differed in their gas production, methane, VFA, A:P and NH3 (Table 6). Accession SA 41020 had significantly (P < 0.05) lower gas (77 kPa), while SA 45574 had significantly (P < 0.05) lower gas (92 kPa), VFA (103) and A:P (2.1) than the others. The accession with the lowest methane production was SA 42690 (35 mL/g) and the highest (twice as much) was SA 45562 (75 mL/g). Ammonia concentrations varied from 378 mg/L (SA 42965) to 614 mg/L (SA 44239).

Table 6. *In vitro* fermentation profiles of 20 accessions of *C. australasicum*

Accession	Gas (kPa)		CH4 (mL/g DM)		VFA (mmol/L)		A:P		NH3 (mg/L)	
SA 4685	103	a	47	cdef	122	a	2.9	a	431	f
SA 4966	108	a	45	defg	124	a	3.0	a	401	hi
SA 41020	77	b	40	fg	125	a	3.0	a	423	fg
SA 41272	102	a	45	defg	117	a	2.9	a	457	e
SA 42690	106	a	35	gh	118	a	3.0	a	491	d
SA 42723	105	a	50	cdef	123	a	3.0	a	488	d
SA 42741	102	a	49	cdef	121	a	3.0	a	454	e
SA 42745	108	a	52	bcde	126	a	3.0	a	449	e
SA 42751	103	a	51	cdef	119	a	3.0	a	525	bc
SA 42766	99	a	50	cdef	121	a	3.2	a	479	d
SA 42772	107	a	50	cdef	121	a	3.1	a	524	bc
SA 42808	99	a	47	cdef	117	a	3.0	a	511	c
SA 42825	102	a	45	defg	118	a	3.0	a	526	b
SA 42965	107	a	47	cdef	119	a	2.9	a	378	j
SA 44239	94	a	53	bcd	124	a	3.1	a	614	a
SA 44286	97	a	53	bcd	125	a	3.0	a	416	g
SA 44383	103	a	57	bc	126	a	3.0	a	462	e
SA 44775	102	a	62	b	120	a	2.9	a	415	gh
SA 45562	105	a	75	a	123	a	2.9	a	412	gh
SA 45574	92	b	63	b	103	b	2.1	b	390	ij
S. E. M.	0.9		0.7		2.2		0.02		5.2	

Significance: within each column, values not sharing the same superscript differ (P< 0.05)

Discussion

Rumen fermentability profiles of selected native perennial shrubs in Australia, as affected by some plant internal and external factors were investigated. Variability in plant fermentation profiles was observed when plants were grown at three different locations in Australia. The largest difference was observed in plant's methane production, with some of the values for individual plants at Monarto being several folds lower than the other two sites. This was also accompanied with reduced gas production as well as acetate : propionate ratios, but not other parameters. It should be noted that plant material from one site (Monarto) for this testing was collected in winter of 2008, compared to samplings from Condobolin and Merredin that were conducted in autumn 2010 in order to keep plants at similar maturity at the time of collection. Another compounding factor that may have influenced the outcome was that for few species, different accessions were used between the sites. While accession SA 44440 of *E. glabra* was used at Monarto, accession SA 45588 was grown at the other two sites, as SA 44440 was unable to be propagated for supply to the other two sites. It was subsequently revealed that these two accessions significantly differ in their methanogenic potential and this may be the reason of this diverse response. However, there were other plants such as *A. saligna* or *E. tomentosa* that were of same accession across the three sites and still produced different responses across the sites. For these, one explanation may be that the environmental conditions such as climate, water and soil conditions may have been such to trigger some specific changes in the plant chemistry, resulting in rather different fermentation profile when fermented

Variability in fermentation parameters was also prominent between the seasons, with values for some accessions nearly doubling in methane production throughout the year. However, there was no clear trend between the seasons, with some values increasing and in some decreasing troughout the year. In addition, plant ranking changed, i. e. some plants that were ranked as 'low' methanogenic in autumn became high methanogenic in other seasons and vice versa. Methanogenic potential of the plants in the rumen may be influenced by many factors, with major drivers being digestibility (nutritive value, NV) and the presence of PSC in the plant. High levels of fibre, for example, stimulate methane production and this may explain why new growth (less fibre) occurring in spring may produce less methane than the older, more fibrous growth in summer (Haddi et al., 2003). Conversely, harsh conditions in summer may induce protective mechanisms such as synthesis of PSC, which in turn may inhibit methane producing microbes in the rumen. As both profiles (NV and PSC) may vary independently between the plants and between the seasons, different mechanisms may be involved in antimethanogenic potential of the plant. This outlines the need for completing NV profiles, but also PSC profiling to identify the mechanisms of these effects.

Literature reports on fermentation kinetics amongst forage shrubs imply of differences between phenological stages of the plant (Haddi et al., 2003). Reproductive parts of the plants may contain more and/or different classes of PSC (Gebrehiwot et al., 2002) and it is also possible that in the other organs PSC change with the growth cycle of the plant. However, in our study, we found only limited differences between phonological stages of the plants, i.e. those in the reproductive phase were not more active than those in the vegetative phase.

In the current study, grazing did not influence overall plant fermentability in any of the four species tested. It is possible that the fresh growth that occurred after grazing improved NV profile of the plant that in turn had beneficial effect on the rumen microbes. Furthermore, it was hypothised that grazing will trigger increased PSC

synthesis in the plants, resulting in their increased bioactivity. However, changes in methane concentrations and the acetate to propionate ratio after grazing were apparent in *A. nummularia* and only to some extent in *E. tomentosa*, while reduction in ammonia occurred with *Rhagodia* species after grazing. It should be noted that some of these plants, i.e. *R. parabolica* cause significant reduction on rumen fermentation even when not grazed. It is therefore possible that some of plants tested here already contain relatively high levels of PSC that in turn deter herbivory, which can also explain low grazing preference for these plants (Jason Emms, pers. comm) and further synthesis of PSC is not required.

The study was also conducted to examine intra-species variability in two focus plants, *E. glabra* and *C. australasicum*. The intra-species variability was observed in both species. In *E. glabra*, the highest methane-producing accession produced three times more methane than the lowest methane producing one, with the strongest inhibitory effects observed with plants that were collected from the wild populations. These differences were also observed in other fermentation parameters, i. e. VFA and gas production. It appears that cultivation of the plant may reduce the need for synthesis of some 'defense' PSC that are also responsible for the bioactive effect and therefore these plants may become less efficient. In *C. australasicum*, there were differences as well, with the lowest methane-producing accession producing half of the methane of the highest producing one.

Conclusions

Environmental conditions may have an impact on plant chemical composition and consequently on plant's effect on rumen microbes, their fermentation pathways and their end-products. Careful examination of plant species x plant accession x location may provide selection of plants that are best suitable for given location to optimize their performance in the rumen of animals grazing these.

It should also be noted that methanogenic ranking of individual plant may also change throughout the year, i.e. plants that are ranked as 'high' methanogenic in one season may become 'low' methanogenic in another season. Variability in methanogenic potential between the seasons may help to assist to develop grazing strategies to reduce methane.

The current findings confirmed our preliminary results about the variable nature and complexity and of plant bioactivity, probably due to variation in synthesis of plant secondary compounds responsible for these effects. Therefore it is necessary to continue screening of plants grown under variable conditions, but also look into specific plant secondary compounds and how they change under these conditions.

In addition, plant bioactivity is a complex issue, relying on plant secondary compounds as well as nutritive composition of the plant. As they may act with different mechanisms in the microbial ecosystem, further investigation in their combined effect will enable selection of plants that may provide a useful tool to manipulate rumen microbial fermentation effectively.

The current findings already offer a choice of several plant x location combinations that may result in low methane output from the animal consuming these plants, however further screening from other locations and different conditions may reveal and provide more of such combinations. This may ultimately provide a selection of plants and conditions that are best suitable for given location to optimize their performance in the rumen of animals grazing these.

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Section 3. Identify possible mechanism(s) and persistency of anti-methanogenic actions from active plants and their compounds

(2 draft manuscripts, 1 short discussion papers)

Draft manuscript 1 - persistency of effects

Dose-dependent antimethanogenic effect of *Eremophila glabra* in batch and Rusitec fermentation

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Abstract

Eremophila glabra, a native Australia shrub, was evaluated for its antimethanogenic activity in both batch and continuous fermentation systems. The batch culture confirms the antimethanogenic effect of *E. glabra*, while the RUSITEC indicates the persistency of this effect under in vitro conditions. Eight doses of *E. glabra* were tested in batch cultures with basal diet (15%, 20%, 25%, 30%, 40%, 50%, 75%, 100%). Three doses were chosen from batch cultures to test in the RUSITEC using 12 fermentation vessels for 21 d (15%, 25%, 40%). Vessels were assigned to three treatments and control with three replicates. When used as supplements in both batch culture and RUSITEC fermentation, *E. glabra* decreased methane production in a dose-dependent manner and altered other rumen fermentation parameters. At high doses (25%, 40%), *E. glabra* seemed to have an adverse effect on rumen fermentation, causing a decrease in total gas production and total volatile fatty acids (VFA) concentrations. However, this inhibition did not occur with the lowest dose. At 15% of the substrate *E. glabra* modified ruminal fermentation by abating methane production without detrimental affect the general fermentation, suggesting that this would be the optimum dose of *E. glabra* for reducing methane emissions from sheep.

Keywords: Batch culture, RUSITEC, Eremophila glabra, methane production

Introduction

Methane produced by livestock contributes approximately 10% of Australian national emission (DCC 2009). Methane is a natural end product of ruminal fermentation and is produced by methanogens. There is evidence that it can be reduced by methane inhibitors, like monensin (Van Nevel & Demeyer 1977 Sep) and bromochloromethane (Goel et al. 2009).

Native plants and their extracts can also be used to modulate ruminal fermentation. Some of them decrease methane in the rumen; for example, *Rheum officinale* and *Frangula alnus* have been shown to decrease methane production and shift volatile fatty acid (VFA) proportions in *in vitro* fermentation (Busquet et al. 2005; García-González et al. 2010; García-González et al. 2008; Durmic et al. 2010). Therefore, there has been an increased interest in native plants and their corresponding extracts for their potential to manipulate rumen fermentation (Wallace et al. 2002; Busquet et al. 2005). Recently, the results from a study of the bioactive properties of Australian native shrub species indicated that leaves of *Eremophila glabra* have a selective effect on ruminal fermentation in batch incubation (Durmic et al. 2010). *E. glabra* produced higher concentrations of propionate and less methane when used as a sole substrate compared with other common pasture species, but there was

some evidence that *E. glabra* affected overall fermentation when used as the sole substrate (Durmic et al. 2010; Li et al. 2010). However, the antimethanogenic effects of *E. glabra* were retained when it was used as mixed substrate with *Medicago sativa in vitro* (Li et al. 2010). This suggests *E. glabra* could be useful as an alternative feed source providing it forms part, rather than the entire diet, of grazing animals (Durmic et al. 2010).

When working with bioactive plants, it is necessary to choose a suitable dose to achieve the most favourable effects (García-González et al. 2008). A dose-response curve for *E. glabra* has not been established but we expect that there is an optimum amount of *E. glabra* that an animal needs to eat to reduce methane production without inhibiting overall fermentation and productivity.

The aim of this study was to determine the optimal dose level(s) of *E. glabra* that would cause a lasting inhibitory effect on methane production without adversely affecting overall ruminal fermentation. Our expectation was that the Rusitec system would reflect the effects we have observed in batch culture and that the effects of *E. glabra* would be persistent. We tested a range of doses of *E. glabra* in batch culture to determine the most suitable doses to test in continuous culture. We used a Rusitec system (continuous culture) to evaluate the persistence of the antimethanogenic effects of *E. glabra* on *in vitro* fermentation.

Materials and methods

The use of animals and the experimental protocol were approved by the Animal Ethics Committee of the University of Western Australia.

Preparation of rumen inoculum

Rumen fluid from five fistulated Merino sheep was collected 2 hours after morning feeding. The sheep were fed on a diet (standard sheep diet, a mixture of 1 part oaten chaff + 0.25 part lupin + 0.025 part minerals) *ad lib* for two weeks before sample collection. The rumen fluid was kept in pre-warmed thermos flasks, strained through 3 layers of cheesecloth, before being used as the source of inoculums.

Batch culture procedures and treatments

E. glabra leaves were harvested from plants in reproductive stage and freeze dried and grounded. The culture substrate consisted of grounded basal diet with a series of 'doses' of *E. glabra*. Prior to the incubation, the culture substrate was weighed into serum bottles and kept in an anaerobic chamber to get rid of oxygen in the bottles. A total of 500 mg of substrate was used in each incubation bottle and a total of 9 substrate treatments:

Control: 500 mg of the basal diet (that was fed to the fistulated sheep),
EG 15%: 75 mg *E. glabra plus* 425 mg basal diet,
EG 20%: 100 mg *E. glabra plus* 400 mg basal diet,
EG 25%: 125 mg *E. glabra plus* 375 mg basal diet,
EG 30%: 150 mg *E. glabra plus* 350 mg basal diet,
EG 40%: 200 mg *E. glabra plus* 300 mg basal diet,
EG 50%: 250 mg *E. glabra plus* 250 mg basal diet,
EG 75%: 375 mg *E. glabra plus* 125 mg basal diet, and
EG 100%: 500 mg *E. glabra*.

Each treatment was incubated in triplicate. The batch culture was conducted in 120 mL serum bottles. The rumen fluid was adjusted to pH 7.2 with McDougall buffer (pH = 8.23) (McDougall 1948), and then 50 mL buffered rumen fluid was added into serum bottles containing the plant substrate. The bottles were sealed with rubber

stoppers and aluminium seals and placed in an incubator for 24 hours at 39°C with constant shaking at 50 rpm.

At the end of the incubation, gas production from the headspace of the bottle was measured using a gas meter (GMH 3110, Greisinger Electronic Gmbh, Germany). A 5 mL gas sample from the headspace of the bottle was withdrawn, kept in a vial and then used to measure methane concentration (in triplicates) in a gas chromatographer (GC, Micro GC Chromatograph, CP-4900, Varian). One mL of the rumen inoculum from each serum bottle was taken, mixed with 200 µL of 1M NaOH and stored at –20□ for analysis of VFA.

Rusitec system and experimental treatments

Two Rusitec fermenters, each equipped with six fermentation vessels (2000 mL overall volume with 1800 mL effective working volume) in a water both, were set up for the experiment following the incubation procedure as described by Czerkawski and Breckenridge (1977). Rumen fluid was collected from the fistulated sheep, pooled and strained through cheesecloth. Five hundred millilitres of the rumen fluid and 1200 mL of artificial saliva (pH 8.23) (Mcdougall 1948) were added into each fermentation vessel. The water bath temperature was set at 39°C. The artificial saliva was prepared daily and infused into the vessels at a rate of 1350 mL/d per vessel. The fermentation vessel was fed daily with 60 g of the feed substrate equally distributed in four nylon bags. In the first 24 h fermentation, one bag containing the solid rumen contents (the solid residue of filtered the rumen fluid, 23 g wet weight) and three bags containing 15 g of the treatment feed substrate were placed in each vessel. From day 2 onwards, the nylon bag containing the rumen solids was replaced with a bag containing 15 g of the feed substrate. The incubation time for each bag of the feed substrate lasted for 48 h. Each vessel was fed daily with 2×15 g bags at 09:30; every day there were two bags taken out and two bags put in to each vessel.

The stability of Rusitec system was assessed by monitoring changes in the pH of the fermentation liquid, measured just before the daily 'feeding' (at 09:30). When the pH fell consistently within the range between 6.49 and 6.53 in all the fermentation vessels, the system was considered to be stable and ready for testing the inclusion of *E. glabra*.

The incubation experiment consisted of two periods: the establishment period of 12 days followed with the treatment period of 21 days. During the establishment period, the feed substrate consisted of solely the basal diet that was fed to the fistulated sheep. The treatment period started on day 13 (referring to day 0 thereafter), and the 12 fermentation vessels were assigned to four feed substrate treatments with three replicates:

Control: basal diet (60 g/d),
EG 15%: 15% inclusion of *E. glabra* (51 g/d basal diet + 9 g/d *E. glabra*),
EG 25%: 25% inclusion of *E. glabra* (45 g/d basal diet + 15 g/d *E. glabra*), and
EG 40%: 40% inclusion of *E. glabra* (36 g/d basal diet + 24 g/d *E. glabra*).

The total amount of *E. glabra* in the treatments was introduced over the first two days. During the first 7 days, the buffer infusion rate to all the vessels was maintained the same and constant. By the end of day 7, the vessels fed 40% inclusion of *E. glabra* were not maintaining what would be considered a normal healthy fermentation and became dark green in colour. This treatment was terminated. It was also noticed that the pH of all the vessels declined noticeably from day 7, so the pH was adjusted and the buffer infusion rate was increased to 1440 mL/d on day 8. Samples were not collected until the system became stable on day 9. Because of this change there were three treatments only from day 9 until day 21.

Sample collection and measurements

The sample collection was started on the last day of the establishment period (referring to day -1), and then throughout the treatment period. The gas that was generated by the fermentation, referring to total gas production, was continuously collected into a wine cask bladder, and the bas was replaced daily before the feeding. The bag was immediately connected to a GC to analyse methane concentration in triplicate. The total gas volume in the bag was measured, and recorded. The outflow of the fermentation liquid was continuously collected into a glass flask, and the flask was replaced daily. The weight of the outflow liquid was recorded. The liquid sample was collected 3 hours after feeding every day, and a 1 mL aliquot was taken and mixed with 200 µL of 1M NaOH and 2M HCl, and stored at –20□ for analysis of VFA.

Chemical analyses

VFA is analysed by gas chromatography using an Agilent 6890 Series GC (Aligent Technologies Inc., Santa Clara, CA, USA) with HP6890 injector, capillary column HP-FFAP, 30m×0.53mm×1.0 µm, FID detector and HP Chemstation software. Hydrogen was used as carrier gas at 6.6 mL/min, with temperatures of the oven T =240 ◦C, injector T =260 ◦C, and detector T =265 ◦C.VFA concentrations in the fermentation liquid were quantified by a capillary gas chromatographer equipped with a split injector and flame ionisation detector (FID), using an internal standard (GC Separation of VFAC2 - C5 Supelco Bulletin No. 749D) calibration method.

Statistical analysis

Results of the batch culture (9 treatments with triplicates) were analysed using the one-way ANOVA procedure of JMP (JMP 5.1.2). Differences between the means were compared using the student t-test and p values < 0.05 were considered significant.

The data from the Rusitec fermentation were analysed according to a randomized block design with repeated measures using the general linear model (GLM) procedure of SAS (SAS Institute Inc., Cary, NC, SAS 9.2) following the statistical model:

$Y_{ijk} = \mu + T_i + Fj_{(i)} + P_k + TP_{ik} + \varepsilon_{ijk}$,

where Y_{ijk} = the dependent variable; μ = the overall mean; T_i = the fixed effect of treatment (i = 0, 15%, 25%, and 40% inclusions of *E. glabra*); $F_{i(i)}$ = the random effect of fermentation vessels (j = 1,…,12) within each treatment i; P_k = the effect of sampling period; TP_{ik} = the interaction of sampling period and treatment; and ε_{ijk} = residual error. Tukey's test was used for multiple comparisons of the means. When a significant (P<0.05) sampling period × treatment interaction was detected, differences among the means were accessed from PROC MIXED by LSD test.

Results

Batch culture

The results of total gas production, methane concentration and production are presented in Table 1. Total gas production was reduced when *E. glabra* was included in the culture substrate at ≥ 40% (P < 0.05). Methane concentration and production started to decline when inclusions of *E. glabra* were > 40 % (P < 0.05). When *E. glabra* was the sole substrate in the batch culture, all measures were significantly lower than those for the basal diet, with 27.2% and 59.0% reductions in total gas and methane production respectively (P < 0.05).

The effects of the level of *E. glabra* inclusion on individual VFAs and total VFA production in batch culture are presented in Table 2. When compared with the

control, the propionate concentration was not influenced by inclusion of *E. glabra* at any level, and the proportion of acetate and total VFA were reduced in the EG 100% treatment (P<0.05). The proportion of butyrate was reduced when the inclusion of *E. glabra* reached > 40%, and the acetate to propionate ratio decreased when the inclusion was > 75% (P<0.05).

Rusitec fermentation

Changes of pH of fermentation liquid

During the first 6 days of the treatment period, the pH of the fermentation liquid was stable for control, and 15% and 25% *E. glabra* inclusions, except for 40% *E. glabra*. The pH value averaged 6.49 with a range between 6.47 to 6.52 for control, and 6.56 (ranged from 6.53 to 6.58) for both 15% and 25% inclusions of *E. glabra*. At 40% inclusion of *E. glabra* the pH increased continuously with time and reached 6.82 by day 5. The pH values in all the vessels started to declien on day 7 to 6.29, 6.39, 6.49 and 6.69, respectively for control, and inclusions of 15%, 25%, and 40% *E. glabra*. For retrieving pH, the buffer infusion rate was increased from 1200 ml/d to 1440 ml/d over days 8 and 9. Therefore, the data for days 8 and 9 were not included in the analysis. The increase of the buffer maintained the pH values stable over the period from day 10 to day 21, averaged at 6.30 for control, and 6.42 and 6.60 respectively for EG 15% and EG 25% treatments.

Dose effects of E. glabra in Rusitec *fermentation over the first 7 days*

The effects of inclusion of *E. glabra* on the fermentation characteristics in the Rusitec for the first 7 days are summarized in Table 3 and Figure 1. Data for days -1 and 0 represent the initial total gas production, methane production and methane concentration when all vessels were 'fed' solely on the basal diet. The total gas production varied from 1543 mL to 1699 mL with a mean methane proportion of 17%. As shown in Fig.1, the inclusion of *E. glabra* in the feed substrate reduced total gas production, methane concentration and production in a dose-dependent manner. There were significant interactions between treatments and days (P<0.05). On day 1, total gas production decreased by 24.4%, 30.9%, 29.5% for EG 15%, 25%, and 40% treatments respectively, when compared to control. However, after day 1, total gas production remained stable at approximately 11.6% and 24.1% lower than the control for the EG 15% and EG 25% respectively. Total gas production decreased to approximately 45% in the EG 40% treatment. A similar trend was observed in methane concentration and production during this period. On day -1 and day 0, 15% inclusion of *E. glabra* persistently reduced methane concentration and methane production (P < 0.05). The 25% and 40 % doses continuously reduced total gas, methane production, and methane concentration over 7 days (P < 0.05). After 24 h incubation with *E. glabra*, based on the changes of total gas production, its effects on overall fermentation became apparent and persisted, but on a dose-dependent manner (Fig. 2).

Mean values of fermentation outputs in 7 days incubation from each treatment are summarized in Table 3. Total gas production and total VFA concentration were not affected by 15% inclusion of *E. glabra*, but methane production was 23.8% lower than the control (P < 0.05). Similarly, vessels supplied with 25% *E. glabra* caused a 22.2% and 27.9% decrease in methane production and gas production separately, compared with control (P < 0.05). In contrast, 25% inclusion of *E. glabra* gave rise to the highest increase of propionate. Methane and gas production were lowest upon 40 % addition of *E. glabra*, which caused a 45.6% and 35.2% decrease separately (P < 0.05). This was accompanied by a reduction in acetate production (-25.6%) and acetate to propionate ratio (-27.0%) (P < 0.05).

Lasting effects of E. glabra on RUSITEC *fermentation over days 10 to 21*

Total gas production, methane concentration and methane production change from each treatment were showed in Figure 3. After stopping 40% inclusion of *E. glabra*, the continuous fermentation lasted for another 14 days. Data from d 8 and d 9 was not included because pH was adjusted on d 8 and stabled on d 9. The effects of *E. glabra* on overall fermentation fluctuated throughout the last 12 days of continuous fermentation (Fig. 3). Supplementation of 15% *E. glabra* inhibited the total gas production on d 11, d 13, d 14, d 20 and d 21 ($P < 0.05$). It had consistently lower methane production and methane concentration than control throughout the whole experimental period, with similar methane values to that observed in the beginning 7 days incubation with 15% addition of *E. glabra* ($P < 0.05$). Vessels supplied with 25% inclusion of *E. glabra* had the lowest total gas production, methane concentration and methane production ($P < 0.05$). However, an unexpected increase in total gas, methane production and methane concentration was observed after d 15, showing no difference to 15% inclusion of *E. glabra* in total gas production from d 17 to d 21 ($P > 0.05$).

Total gas production from vessels in the EG25% treatment increased on day 16, so the analysis was separated into 2 periods from d 10 to d 21 (period 1, d 10 to d 15; period 2, d 16 to d 21). Mean values of total gas, methane and VFA productions from each treatment in both periods are presented in Table 4. On average, inclusion of 15% *E. glabra* did not affect total gas production in either period and total VFA production in period 1. Methane concentration and methane production, the proportion of acetate, propionate and butyrate, and acetate to propionate ratio were reduced in both periods ($P < 0.05$). EG25% reduced all VFA parameters throughout the collection period ($P < 0.05$), with the exception propionate production, which was highest in this treatment ($P < 0.05$). Total gas (29.9%) and methane production (72.7%) increased significantly in period 2 in EG25%.

Discussion

Plant secondary compounds can be used as feed additives to modulate ruminal fermentation, especially to reduce methane production from livestock (Patra & Saxena 2010). Alternatively, direct use of the plants that contain those compounds can produce similar effects on fermentation patterns (Alonso-Diaz et al. 2010). A Rusitec system was used in this study to confirm the persistence of the antimethanogenic effects of *E. glabra* and to select an optimal dose to feed animals. In both batch and Rusitec studies, the decrease in methane production in response to the addition of *E. glabra* in the basal diet confirmed results earlier experiment in batch culture (Durmic et al. 2010; Li et al. 2010). The reduction in methane production was persistent and positively related to the dose of *E. glabra*. Total gas production was lower at higher doses suggesting that there was a general inhibitory effect on ruminal microorganisms. However, at low doses (EG15%), there appeared to be a more specific effect on methanogenesis because the other indicators of 'normal' rumen fermentation were not affected. The EG15% treatment seems to be the optimal dose to feed animals to obtain the antimethanogenic benefits of *E. glabra* without causing detrimental effects on rumen fermentation.

Testing different doses of *E. glabra* in batch culture confirmed our results from an earlier study where we demonstrated that *E. glabra* maintains its antimethanogenic potential when it is mixed with other forages (Li et al. 2010). The batch culture system is a cost effective way to compare the effects of different doses of *E. glabra,* but data from short-term *in vitro* fermentation studies may over-estimate the modulation effect. Ruminal microbes can build up resistance to additives over the longer term so it is important to test whether these effects persist (Cardozo et al. 2004).

We used the results from the batch culture experiment to guide our choice of dose to include in the Rusitec study. The lowest but effective dose of *E. glabra* in batch culture was 50% (0.25 g *E. glabra* in 50 mL rumen fluid), which is equivalent to 5 g/L of *E. glabra* in the fermentation media and 15% of the substrate added to each vessel of the Rusitec (9 g *E. glabra* in 1.8 L rumen fluid). We also included higher doses (25% and 40%) because the batch culture tends to over-estimate the effects on fermentation and to confirm whether the overall inhibitory effects we had observed in batch culture were less potent in the continuous culture system, where there is constant buffering and removal of end products.

The Rusitec systems enables a stable, long-term, fermentation to be undertaken and enables the adaptation of ruminal microorganisms to substrates to be examined over a period of several weeks (Wallace et al. 1981). Ruminal microbes can adapt to some of the additives after several days incubation (Busquet et al. 2005; Cardozo et al. 2004) and could minimise the positive modulation effects *E. glabra* has on ruminal fermentation. Before planning a costly *in vivo* experiment, it is essential and economic to evaluate any possible adaptation effects of ruminal microbes beforehand. It is been suggested that 2 weeks incubation with supplements is an adequate period to observe any adaptation of microbes to plant supplements in the Rusitec (Goiri et al. 2009).

Methane production was reduced in all *E. glabra* treatments tested in the Rusitec. The antimethanogenic effect was dose-dependent and prolonged for 21 days of fermentation, indicating that *E. glabra* is resistant to adaptation of methanogens over this period of time. The methane reduction occurred at lower doses in continuous fermentation compared with batch culture, with *E. glabra* reduced methane production at lower doses in Rusitec fermentation (15%, 25%, 40%) than in batch fermentation (≥ 50%), compared with control. It is possible that the daily addition of *E. glabra* substrate to the Rusitec had a greater influence on methane reduction than when it was added as a single dose over 24 hours in the batch culture system. These results confirm the need to examine the effects of promising candidates identified in batch culture in a longer-term continuous culture system.

Total gas production and VFA concentrations are used to reflect fermentation kinetics. Generally, overall fermentation was not affected by 15% inclusion of *E. glabra* over a 21 day period, indicating that the inclusion of *E. glabra* at 15% of the substrate did not affect total ruminal microbes. In contrast, the EG25% and EG40% depressed total gas production throughout the entire experimental period, suggesting that there might be a decrease in the ruminal microbial population or an inhibition on their activity. Total gas and methane production increased slightly in the last 5 days of fermentation in EG25%. This suggests there may have been some adaptation to *E. glabra* but it may also be because there is evidence for treatments to converge in long-term Rusitec studies. However, it will be important to examine the persistence of the effects *in vivo* to confirm whether true adaptation is occurring or if it is an anomaly of the Rusitec system.

E. glabra has the potential to shift the fermentation in the rumen towards more efficient pathways with higher propionate production, lower acetate and butyrate production and less methane. However, at the higher doses (EG25% and EG40%) this effect was accompanied with a decrease in total VFA production, is detrimental and an indication of general inhibition of rumen microbial fermentation. All treatments caused similar shifts in the proportions of VFAs produced. Propionate proportion was increased with all doses, but not in a dose-dependent manner. The highest propionate proportion occurred with EG25%, suggesting that 25% addition could be a maximum supply level between beneficial effects and detrimental effects on rumen

fermentation. An increase in propionate production at the expense of methane indicates a favourable shift in hydrogen utilisation. The effects of different doses on

It is clear from our results that *E. glabra* had a dose-dependent and persistent antimethanogenic effect in *in vitro* continuous culture fermentation. The microbial community did not appear to adapt to *E. glabra* after 21 d of fermentation when it was included as 15% of the substrate. A substantial inhibition of fermentation was observed with the 40% dose, but at 15%, *E. glabra* may appeared to act primarily to inhibit methanogens in the rumen. A closer look at the methanogens and cellulolytic bacterial populations using molecular analysis could explain the mechanism behind the methane reduction. It has been suggested that data from short-term *in vitro* fermentation studies should be confirmed in long-term continuous culture fermentations, before attempting to undertake *in vivo* experimentation. Our results would support this suggestion because the same concentration of *E. glabra* in the Rusitec fermentation caused slightly different changes in fermentation patterns compared to batch cultures, and the effects occurred at lower doses. Based on our results, including *E. glabra* at 15% of the diet would be an optimal level to feed animals to reduce methane production, without adversely affecting rumen fermentation.

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Table 1. Effect of different doses of *E. glabra* inclusion on total gas production, methane concentration and methane production in batch culture

	Total gas production (mL/ g)	CH_4 (%)	CH_4 Production (mL/ g)
Basal diet	215±2.81^{a}	11.9±0.20^{ab}	25.5±0.71^{a}
EG 15%	213±1.14^{a}	11.9±0.07^{ab}	25.4±0.28^{a}
EG 20%	213±0.86^{a}	12.0±0.50^{ab}	25.5±1.14^{a}
EG 25%	209±2.85^{ab}	11.0±0.46^{ab}	23.1±1.17^{ab}
EG 30%	211±1.39^{ab}	11.6±0.15^{ab}	24.5±0.46^{ab}
EG 40%	204±0.79^{b}	11.0±0.29^{ab}	22.4±0.67^{ab}
EG 50%	202±2.76^{b}	10.6±0.11^{bc}	21.4±0.47^{b}
EG 75%	192±1.08^{c}	9.3±0.11^{c}	17.8±0.29^{c}
EG 100%	184±0.89^{c}	7.8±0.18^{d}	14.3±0.38^{d}

Values are the mean ± standard error of means.
$^{a-d}$ Values in the same column with different superscript letters are significantly different at $P < 0.05$.

Table 2. Effect of different doses of *E. glabra* inclusion on concentrations of individual VFAs and total VFA in batch culture

	Acetate (mmol/L)	Propionate (mmol/L)	Butyrate (mmol/L)	Total VFA (mmol/L)	A:P
Basal diet	72.2±2.18^{a}	27.3±0.76^{a}	11.5±0.30^{a}	112±3.27^{a}	2.64±0.015^{a}
EG15%	71.7±0.18^{a}	27.1±0.08^{a}	11.3±0.02^{ab}	111±0.25^{ab}	2.65±0.001^{a}
EG20%	72.2±0.30^{a}	27.0±0.11^{a}	11.3±0.08^{ab}	112±0.49^{ab}	2.67±0.001^{a}
EG25%	69.7±0.62^{ab}	26.3±0.14^{a}	11.1±0.09^{ab}	108±0.84^{ab}	2.65±0.010^{a}
EG30%	72.0±1.20^{a}	27.1±0.33^{a}	11.5±0.17^{a}	112±1.69^{ab}	2.65±0.014^{a}
EG40%	71.0±0.75^{ab}	26.7±0.23^{a}	11.1±0.11^{ab}	110±1.10^{ab}	2.65±0.006^{a}
EG50%	69.3±1.08^{ab}	26.4±0.32^{a}	10.6±0.14^{b}	107±1.52^{ab}	2.62±0.021^{a}
EG75%	68.9±1.54^{ab}	28.5±0.74^{a}	9.0±0.21^{c}	107±2.49^{ab}	2.42±0.015^{b}
EG100%	65.3±1.60^{b}	28.6±1.04^{a}	8.0±0.17^{d}	103±2.84^{b}	2.29±0.027^{c}

Values are the mean ± standard error of means.

[a-d] Values in the same column with different superscript letters are significantly different at $P < 0.05$.

Table 3. Effects of three addition levels of *E. glabra* on ruminal fermentation outputs in RUSITEC for the first 7 days

Treatment	Control	EG 15 %	EG 25%	EG 40%	s.e.m.
Total gas production, mL/d	1666 [a]	1492 [a]	1234 [b]	1018 [c]	111
CH_4, %	17.9 [a]	13.1 [b]	12.2 [b]	8.8 [c]	1.31
CH_4, mL/d	304 [a]	196 [b]	157 [bc]	104 [c]	26.7
Total VFA, mmol/L	105 [a]	98 [ab]	96 [b]	79 [c]	3.00
Acetate, mmol/L	64.1 [a]	59.1 [b]	56.8 [b]	45.5 [c]	1.80
Propionate, mmol/L	24.1 [b]	24.3 [b]	27.5 [a]	23.3 [b]	0.92
Butyrate, mmol/L	17.5 [a]	15.0 [b]	12.3 [c]	9.8 [d]	0.69
a : p	2.8 [a]	2.4 [b]	2.1 [c]	1.9 [c]	0.06

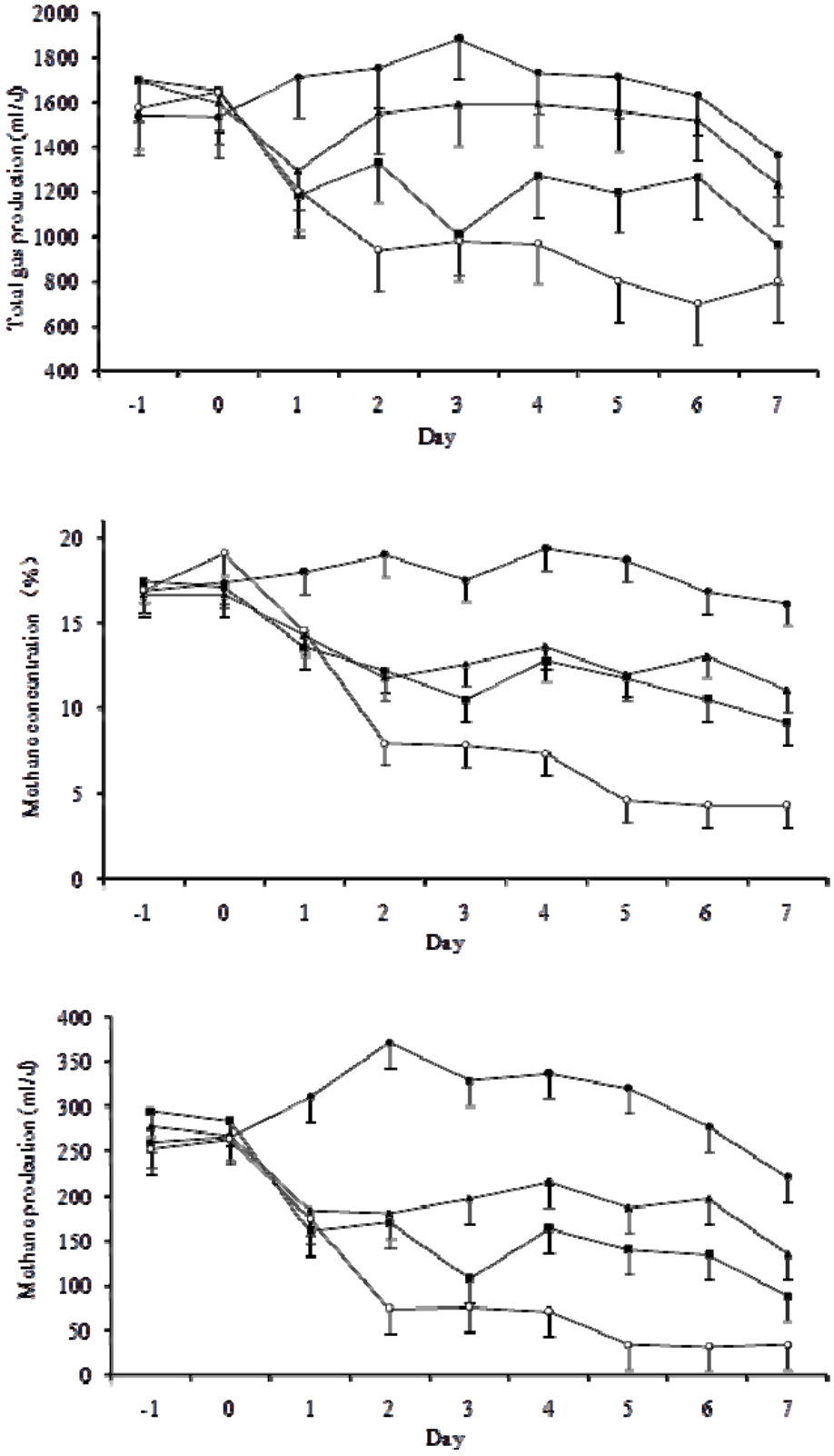
[a-c] Values in the same row with different superscript letters are significantly different at $P < 0.05$.

Table 4. Effects of 15% and 25% inclusions of *E. glabra* on Rusitec fermentation from day 10 until day 21

	Control	EG 15%	EG 25%	s.e.m.
Total gas production, mL/d				
Days 10 to 15	1723 [a]	1491 [a]	1014 [b]	92.6
Days 16 to 21	1634 [a]	1457 [ab]	1317 [b]	92.6
Days 10 to 21				
Methane, mL/d				
Days 10 to 15	297 [a]	174 [b]	66 [c]	23.9
Days 16 to 21	251 [a]	160 [bc]	114 [c]	23.9
Days 10 to 21				
CH4, %				
Days 10 to 15	17.1 [a]	11.7 [b]	6.4 [c]	0.86
Days 10 to 21				
Total VFA, mmol/L				
Days 10 to 15	113 [a]	106 [a]	96[b]	3.33
Days 16 to 21	106 [a]	97 [b]	81 [c]	2.97
Days 10 to 21				
Propionate, mmol/L				
Days 10 to 15	24.6 [c]	28.4 [b]	34.8 [a]	0.94
Days 16 to 21	23.5 [c]	25.3 [b]	27.2 [a]	0.84
Days 10 to 21				
Acetate, mmol/L				
Days 10 to 15	68.3 [a]	60.2 [b]	53.1 [c]	2.22
Days 16 to 21	63.4 [a]	54.8 [b]	46.7 [c]	1.98
Days 10 to 21				
Butyrate, mmol/L				
Days 10 to 15	19.6 [a]	17.5 [b]	8.2 [c]	0.59
Days 16 to 21	18.8 [a]	16.7 [b]	6.8 [c]	0.57
Days 10 to 21				
a : p				
Days 10 to 15	2.77 [a]	2.12 [b]	1.52 [c]	0.05
Days 16 to 21	2.70 [a]	2.16 [b]	1.73 [c]	0.05
Days 10 to 21				

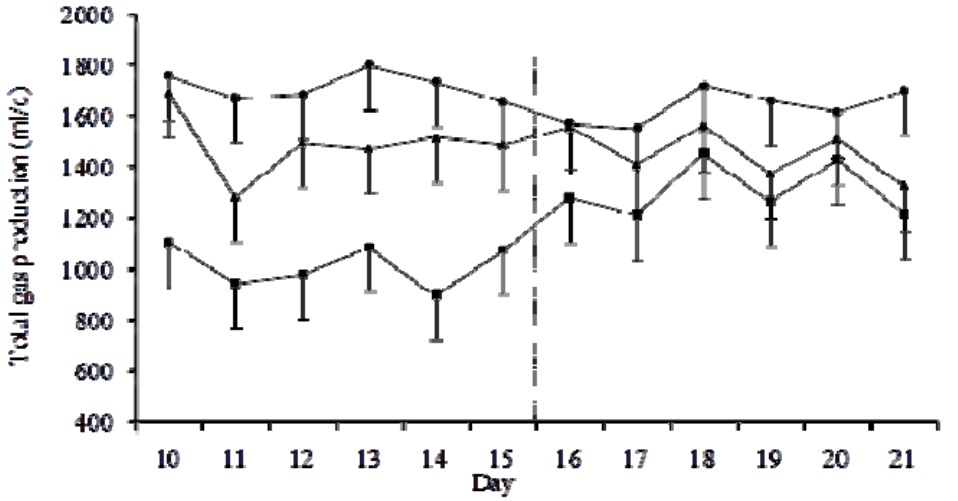
[a-c] Values in the same row with different superscript letters are significantly different at $P < 0.05$.

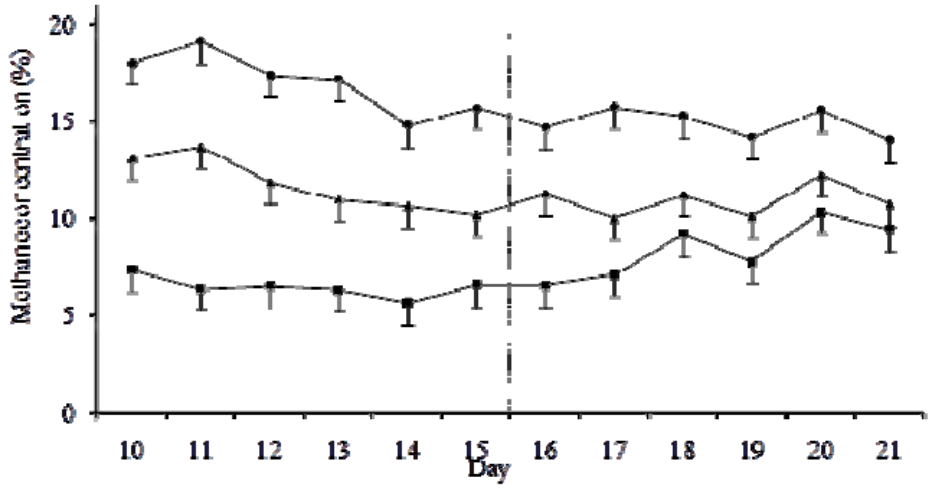
Figure 2. Total gas production, methane production and methane concentration in vessels fed with control (●), 15%, (▲), 25% (□), and 40% (○) inclusion of *E. glabra* from day -1 to day 7. The error bars represent the standard error of the

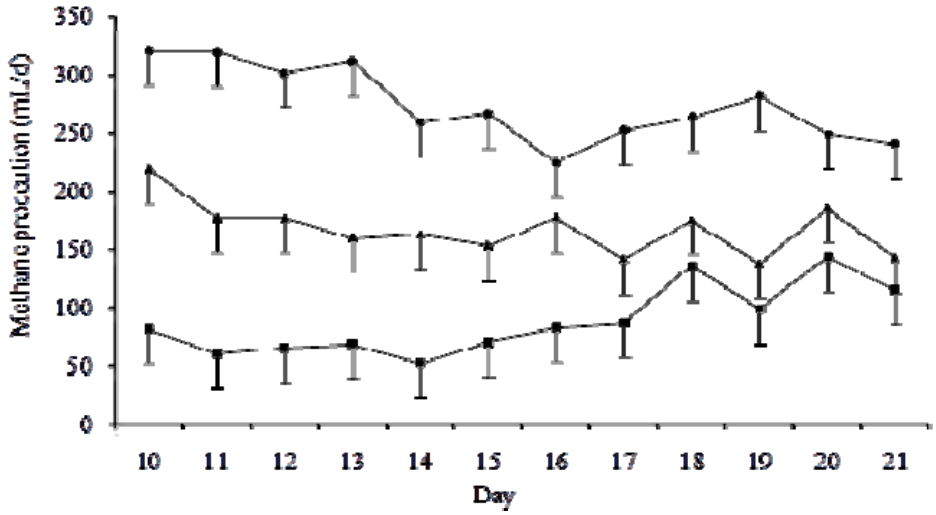


means

Figure 3. Total gas production, methane production and methane concentration in vessels fed with control (●), 15%, (▲), and 25% inclusion of *E. glabra* (□) from day 10 to day 21. The error bars represent the standard error of the means


Total gas production (ml/g)
2000
1800
1600
1400
1200
1000
800
600
400
10
11
12
13
14
15
16
17
18
19
20
21
Day


Methane concentration (%)
20
15
10
5
0
10
11
12
13
14
15
16
17
18
19
20
21
Day


Methane production (mL/d)
350
300
250
200
150
100
50
0
10
11
12
13
14
15
16
17
18
19
20
21
Day

Draft manuscript 2 – mechanism of action at microbial level

Inhibition of ruminal methanogens and selected cellulolytic bacteria during fermentation of *Eremophila glabra* in RUSITEC

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Abstract
Eremophila glabra was tested as a part of a mixed diet in RUSITEC continuous culture system. Twelve fermentation vessels were used to evaluate the effect of 3 doses of *E. glabra* (15%, 25%, and 40%) on rumen methanogens, rumen bacterial numbers and in particular cellulolytic bacteria over a 34 d period. After an adaptation period on control diet, of 12 days, *E. glabra* was introduced in the system. Samples were collected 24 hr before inclusion of *E. glabra* and then every five days until the end of the trial. Samples were analysed for total numbers and types of methanogens using real-time PCR (qRT-PCR) and specific primers. The total number of methanogens was assessed using primers towards the methyl coenzyme M reductase gene (mcrA), while types of methanogens were assessed using probes to the 16S rDNA gene. The total number of methanogens decreased significantly by 28.8%, 38.0%, 42.1% in the 15%, 25%, 40% *E. glabra* treatments respectively. The diversity of methanogens was also was affected and shifted towards *Methanobrevibacter spp.* when the system was exposed to *E. glabra*. *Fibrobacter succinogenes* was inhibited in all doses, while *Ruminococcus albus* was only inhibited at the highest dose of *E. glabra*. When *E. glabra* is included at 15% of the diet there is a positive effect on rumen fermentation that seems to be persistent. However, the inhibition of *F. succinogenes*, one of the key bacterial species involved in cellulose hydrolysis in the rumen, needs to be investigated further *in vivo*, to establish if there is an effect on digestibility and other production parameters before any firm conclusions can be made about the role of *E. glabra* in new grazing systems.
Key words: qPCR, Rusitec, Methanogens, Cellulolytic bacteria

Introduction
Methane formation from ruminants is a major contributor to greenhouse gas within the agriculture system in Australia (DCC 2009). Methane produced during the anaerobic enteric fermentation of feed can be affected by components of the diet components, and lead to a shifts in the ruminal microbial population (Fernando et al. 2010). Plants with bioactive properties have been shown to reduce methane production *in vitro* (Durmic et al. 2010). These bioactive compounds can decrease methane production through a direct inhibition of methanogens or indirectly by affecting other microorganisms in the rumen that are responsible for hydrogen production and utilisation, for example the cellulolytic bacteria (Animut et al. 2008).

Eremophila glabra is an Australian native plant that has the potential to reduce methane by rumen microbes, but also affects overall microbial fermentation (Durmic, 2010a) . These effects occurred in batch culture and when *E. glabra* was included as a mixed substrate (25 g per 100 g substrate) in RUSITEC (Durmic, 2010b) and was

associated with reduction in rumen methanogens (A.Wright, pers. comm.). In a subsequent study, we found that when *E. glabra* is included a 15%, 25% and 40% of the substrate in the RUSITEC, the antimethanogenic effects are persistent and dose-related. At 15%, methane production was reduced without affecting overall gas production, whereas at the higher doses, there was an overall inhibition of fermentation. These results suggested *E. glabra* may have selective inhibitory effects at lower doses and more general inhibitory effects at higher doses. We tested this hypothesis by examining the effects of different levels of *E. glabra* on selected rumen microbes during RUSITEC fermentation.

Materials and methods

Experimental design

This study was conducted on samples collected from a previous study (Li et al. 2011; Section 3 this report). Briefly, a RUSITEC experiment was conducted as described previously (Li et al. 2011). Twelve fermentation vessels (2000 mL volume, 1800 mL of effective working volume, in 39°C water bath) were set up and allowed to acclimatize for 12 days on a basal diet (Li et al. 2011).

After acclimatization, *E. glabra* was introduced daily into the vessels at 15%, 25% and 40% of the substrate provided in each vessel and the system was run for another 21 days. The treatments were done in triplicate. The 40% inclusion of *E. glabra* was terminated after 7 days because the overall fermentation was inhbited.

Rumen fluid samples for rumen microbial profiling were collected from each fermentation vessel the day before starting the *E. glabra* treatments, and then every five days until the completion of the trial. The samples (10 mL each) were taken 3 h after changing the feed bags and placed into sterile tubes and stored at -20°C.
A quantitive real-time PCR (qRT-PCR) assay was used to estimate the numbers and population changes of ruminal methanogens and three major ruminal cellulolytic bacteria: *Fibrobacter succinogenes* (gram-negative), *Ruminococcus albus* and *Ruminococcus flavefaciens* (gram-positive). These bacteria were selected as markers for the effect on overall microbial populations.

DNA extraction

DNA extraction was carried out using a modified method of Denman and McSweeney (2006). In general, the genomic DNA was extracted using a bead-a beating method and QIAamp DNA Mini Kit (Qiagen) and the Fast Prep Instrument (Q-BIO gene, Quebec, Canada) (Denman & McSweeney 2006). Total genomic DNA was isolated from a 2 mL aliquot of the 10 mL fluid samples taken from the RUSITEC. The sample was added into a screw-capped microcentrifuge tube using a wide-bore pipette, and centrifuged at 14 000 rpm for 2 min. The supernatant was removed and pellet was combined with bead mix (200 mg) and and lysis buffer (200 µL), vortexed and placed in a bead beater (Fast Prep Instrument). Following this, the sample was incubated at 70°C for 15 min and then centrifuged for 5 min at 14 000 rpm. The supernatant (180 µL) was transferred into a new eppendorf tube containing 20 µL proteinase K and incubated for 3 h at 56°C.

qRT-PCR primers and assay conditions

The primers used for the qRT-PCR are listed in Table 1. qRt-PCR assays for enumeration of total bacteria, methanogens, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* were performed according to the methods described by Denman and McSweeney (2006) and Denman *et al*.(2007) on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, USA?).

Briefly, qRt-PCR assays were performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using a Platinum SYBR green quantitative PCR Super Mix-UDG (Invitrogen, USA). Assays were set up using the reaction mixture (35 µL) comprised of a Platinum SYBR green mix (17.5 µL), the primer (forward and reverse,3.5 µL each), Rox (0.7 µL), distilled water (8.3 µL) and the DNA template (5 µL). The quantitative PCR assays were conducted with the following cycle conditions: one cycle at 50°C for 2 min, and 95°C for 2 min for initial denaturation; 40 cycles at 95°C for 15 s and 60°C for 1 min for primer annealing and product elongation (Denman et al. 2007). The dissociation curve analysis of PCR end products was performed at 95°C for 2 min and 60°C for 15 s. Total microbial DNA was diluted to 1:20 prior to use in real-time PCR assays.

Table 1. PCR primers used for real-time PCR assay

Target species	Forward primer	Reverse primer
Total bacteria	CGGCAACGAGCGCAACCC	CCATTGTAGCACGTGTGTAGCC
mcrA	TTCGGTGGATCDCARAGRGC	GBARGTCGWAWCCGTAGAATCC
Fibrobacter succinogenes	GTTCGGAATTACTGGGCGTAAA	CGCCTGCCCCTGAACTATC
Ruminococcus flavefaciens	CGAACGGAGATAATTTGAGTTTACTTAGG	CGGTCTCTGTATGTTATGAGGTATTACC
Ruminococcus albus	CAAAACCCTAAAAGCAGTCTTAGTTCG	GACGGGCGGTGTGTACAAG

Amplification efficiencies for the methanogens and total bacteria qPCR assay were determined on plasmid DNA as described by Denman (2007). The copies of these microbes were calculated based on their DNA concentrations and base pair sizes and used in a 4 log dilution for methanogens.

Standard curves for the absolute quantification of *F. succinogenes*, *R. flavefaciens* and *R. albus* were derived using the method of Denman and McSweeney (2006). The copies of these microbes were calculated based on their DNA concentrations and base pair sizes and used in a 8 log dilution series.

Statistical analysis

Data were analysed using the GLM procedure of SAS (SAS Institute Inc., Cary, NC, SAS 9.2). Microbial numbers were transformed to a $\log_{10}$ scale prior to analysis. In the mixed model, time (i.e., day of sampling) and treatment were set as fixed effects and fermenters as random effect. Tukey's test was used for the multiple comparisons of the means. When the time × treatment interaction was significant, differences

among the means were accessed from PROC MIXED by LSD test. Significance and trends were declared at $P < 0.05$.

Results

Total bacterial numbers

The total numbers of bacteria ranged from $5x10^9$ to $8x10^9$ cfu/mL. There were no significant differences throughout the incubation period.

Methanogen numbers and profiles

The numbers and profiles of the methanogen population present in the *E. glabra* treatments and control are presented in Table 2. The changes in the population in comparison to the control are shown in Fig 1.

Table 2. Absolute number of methanogens quantified by real-time PCR. The values were log_{10} transformed (mean±SEM log_{10} cells/mL)

Day	Control	EG 15%	EG 25%	EG 40%
-1	8.03±0.07	7.99±0.06	8.12±0.01	8.04±0.08
1	8.21±0.03^{a}	7.94±0.04^{b}	7.82±0.10^{b}	7.76±0.02^{b}
6	8.11±0.03^{a}	8.11±0.06^{ab}	8.10±0.13^{b}	7.72±0.01^{c}
11	8.36±0.03^{a}	8.19±0.02^{b}	7.96±0.07^{c}	
16	8.41±0.06^{a}	8.27±0.05^{b}	8.27±0.00^{b}	
21	8.52±0.04^{a}	8.25±0.06^{b}	8.29±0.05^{b}	NM

$^{a-c}$ means in a same row with different superscript letters are significantly different at $P < 0.01$

NM – not measured because 40% treatment was stopped on day 7

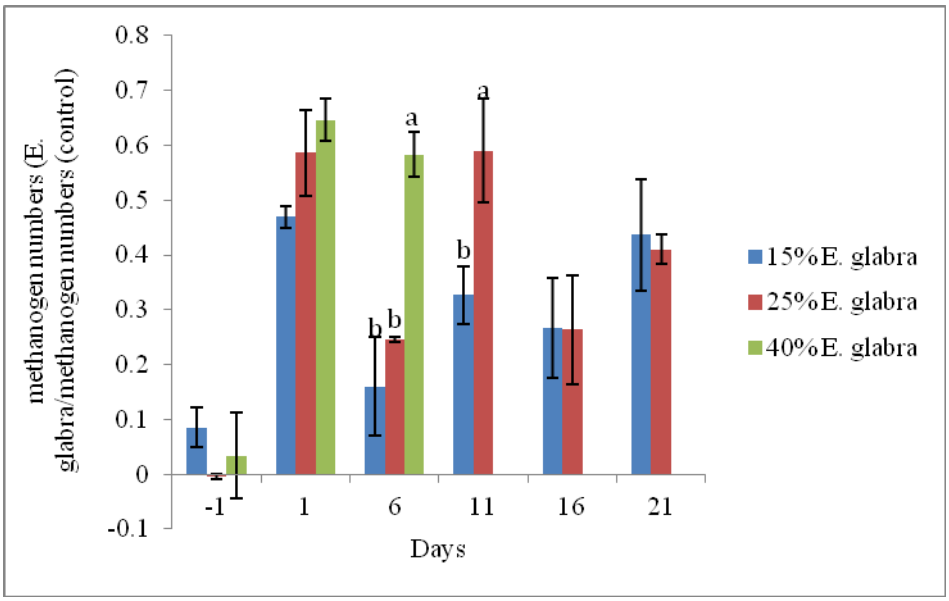

Fig.1 Relative changes in methanogen numbers during incubation with different levels of *E. glabra* when compared to the control. ab - means in a sampling points with different letters are significantly different at $P < 0.01$.

Inclusion of *E. glabra* at all three levels resulted in an immediate decrease in methanogen populations by 47.0%, 58.7% and 64.7% respectively ($P < 0.01$), but the decrease was not closely related to the doses of *E. glabra* (Fig 1.). Over the time of

the trial, numbers of methanogens remained significantly reduced, with an overall average reduction in the total number of methanogens of 28.8%, 38.0%, 42.1% for EG15%, EG25% and EG40% respectively.

Dissociation curve analysis of the amplicons revealed two peaks clearly identifiable at 81°C and 86°C in the control. However, there was an increase in the 81°C dissociation peak and a reduction in the 86°C dissociation peak in the *E. glabra* treatments.

Cellulolytic bacterial population numbers and profiles

The effects of *E. glabra* on the numbers of key cellulolytic bacteria in the rumen (*F. succinogenes* and *R. albus*) are shown in Tables 3 and 4. The population of *R. flavefaciens* in the samples was below the detection threshold for analyzing DNA concentration by qRt-PCR, so no results are reported here.

On the basal diet (ie, day -1) there were no significant differences in populations of *F. succinogenes* and *R. albus* between the fermentors. After 24 h of incubation with *E. glabra, F. succinogenes* populations were significantly reduced in all *E. glabra* treatments ($P < 0.01$, Table 3) and in a dose-dependent manner. The effects were consistent until day 6, with the populations of *F. succinogenes* in EG15%, EG25%, and EG40% about 10, 100, and 1000 fold lower than the initial population in corresponding vessels. In *E. glabra* treatments and from day 11 onwards, *F. succinogenes* populations for 25% *E. glabra* remained significantly lower than the control ($P < 0.01$ Table 3), and the populations for 15% *E. glabra* were lower than the control but not significant on days 16 and 21 ($P > 0.01$, Table 3).

Table 3. Absolute number of *F. succinogenes* quantified by real-time PCR. The values were log_{10} transformed (mean±SEM log_{10} cells/mL)

day	Control	EG 15%	EG 25%	EG 40%
-1	6.28±0.07	6.27±0.14	6.30±0.11	6.31±0.07
1	6.31±0.06^{a}	5.21±0.25^{b}	4.24±0.08^{c}	3.82±0.21^{c}
6	6.35±0.12^{a}	5.46±0.25^{b}	4.29±0.58^{c}	3.31±0.02^{d}
11	6.02±0.03^{a}	4.94±0.19^{b}	4.58±0.48^{b}	
16	5.85±0.09^{a}	5.15±0.13^{ab}	4.40±0.06^{b}	
21	6.07±0.04^{a}	5.01±0.09^{ab}	4.43±0.06^{b}	NM

Values are the mean ± standard error of means from three vessels per treatment and triplicated analysis per vessels.

$^{a-c}$ means in a same row with different superscript letters are significantly different at $P < 0.01$.

NM – not measured because 40% treatment was stopped on day 7

The changes in the *R. albus* population did not show any clear patterns during the whole incubation period, and the population fluctuated with time within each of the treatments. Significant reductions in the population were observed in the 40% *E. glabra* inclusion compared with the other treatments ($P < 0.05$), between 25% and 15% *E. glabra* levels on day 6, and between the control and 25% *E. glabra* treatment on day 21 (Table 4).

Table 3. Absolute number of *R. albus* quantified by real-time PCR. The values were log_{10} transformed (mean±SEM log_{10} cells/mL)

day	Control	EG 15%	EG 25%	EG 40%
-1	7.33±0.11	7.34±0.09	7.40±0.02	7.09±0.17
1	7.30±0.05	7.41±0.08	7.43±0.13	7.51±0.10
6	7.27±0.13^{ab}	7.48±0.07^{a}	7.11±0.14^{b}	6.67±0.12^{c}
11	7.14±0.10	6.86±0.03	6.88±0.08	
16	7.09±0.09	7.25±0.07	7.09±0.18	
21	7.08±0.07^{b}	7.29±0.08^{ab}	7.43±0.12^{a}	

Values are the mean ± standard error of means from three vessels per treatment in triplicate.

$^{a-c}$ means in a same row with different superscript letters are significantly different at $P < 0.01$.

Discussion

Our hypothesis was that *E. glabra* may have selective inhibitory effects on methanogens at lower doses and more general inhibitory effects at higher doses. This hypothesis was based on the emissions profiles we observed when the three doses of *E. glabra* were tested in the tested in the Rusitec (reported in Li et al.2011; Section 3, this report). Our results support this hypothesis despite F. succinogenes, one of the key cellulolytic bacteria in the rumen, being inhibited at all doses of *E. glabra*. We have demonstrated that the inclusion of *E. glabra* as a part of a mixed diet in the RUSITEC decreased the methanogen population significantly. The effect was immediate and persisted for the duration of the study (three weeks). These results support our previous studies where only one level of inclusion was tested (25 g/100 g control diet) and we detected a significant decrease in methane and numbers of methanogens (Durmic, 2010b).

In the current study, the inhibitory effect on methanogens was not dose-dependant, and higher inclusion levels did not have higher inhibitory effects. In fact, in our recent study (Li et al. 2011) at 15% dose, methane production was significantly lower than the other doses, indicating that the activity of methanogens was also affected in non-linear manner. This is consistent with the idea that methanogen population density is not linearly related to enteric methane emission (Zhou et al. 2011). The specific methanogen species, rather than the total methanogens, have a greater effect on methane production (Zhou et al. 2011).

In this study, *E. glabra* caused a shift in the methanogen communities. Dissociation curves can be used to examine changes in population structure. The two distinct peaks observed in the dissociation curve of the real-time PCR amplicons revealed the cluster with *Methanobrevibacter spp.* at 81°C, while the second peak at 86°C corresponded to other methanogenic orders (Denman et al. 2007). *Methanobrevibacter ruminantium* gradually became the major methanogen species in the fermentors where *E. glabra* was present as substrate treatments. *Methanobrevibacter* appears to have a higher tolerance to *E. glabra* and had a competitive advantage over other methanogen species in the presence of *E. glabra*. We can speculate that the shift in the methanogen population towards higher numbers

of *Methanobrevibacter* was responsible for the reduction in reduction in methane. It is important to confirm this and investigate what gives *Methanobrevibacter* the ability to tolerate the bioactivity of *E. glabra.* It would be helpful to use a gradient gel electrophoresis to accurately confirm this shift in methanogen diversity *in vivo*.

The inclusion of this *E. glabra* at all levels reduced the population of *F. succinogenes,* which may have resulted in a lower amount of metabolic hydrogen being produced and consequently less methane production. *F. succinogenes.* In contrast, another cellulolytic bacteria, *R. albus*, was not affected and was even stimulated at some time points in EG15% and EG25%. This finding was unexpected because the common bioactive compounds in *E. glabra,* Serrulatane diterpenes are toxic to gram-positive bacteria as opposed gram-negative species (Ndi et al. 2007). This suggests that compounds other than Serruletane diterpenes in *E. glabra* are responsible for the rumen modulating effects that we have examined. This supports someour preliminary examination of fractions that we have purified from *E. glabra* and reported elsewhere (Li et al, 2011; Section 3, this report). It is important to note that the DNA samples were extracted from the incubation liquid, and not the solid feed residue, and since cellylolytic bacteria tend to adhere to particulate matter, these populations may have been underestimated in the current study. There is a clear need to investigate further the effect *E. glabra* has on other rumen microbes in pure culture as well as *in vivo*. We have designed an in vivo experiment based on the results reported here, where we will investigate the effects of *E. glabra* on methane production, digestibility and microbial ecology when animals are fed a diet consisting of 15% *E. glabra*.

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Short discussion paper 1: Specific or general antimicrobial activity

Background

Ethanolic extracts were obtained from 12 plants (Figure 1) and tested at 500 µg/mL against pure cultures of four representative rumen methanogenic species: *Methanobrevibacter gottschalkii, M.ruminantium, Methanobacterium bryantii* and *Methanosarcina barkerii.* Methane production by cultures in the presence of the extracts was measured and compared to methane production by cultures without addition of extracts to estimate the inhibitory effect of the extract on pure cultures of methanogens.

Results

Plant extracts varied in their effect on pure cultures of methanogens. There were those that caused complete inhibition (100%) of all four methanogens, i.e. *Eucalyptus occidentalis* and *E. glabra* PH and others like *Acacia baileyana*, *A. saligna* and *E. glabra* S212 that completely inhibited only selected methanogens. The level of inhibition with other plant extracts ranged from no inhibition to 60% inhibition, again with variable response in different methanogens.

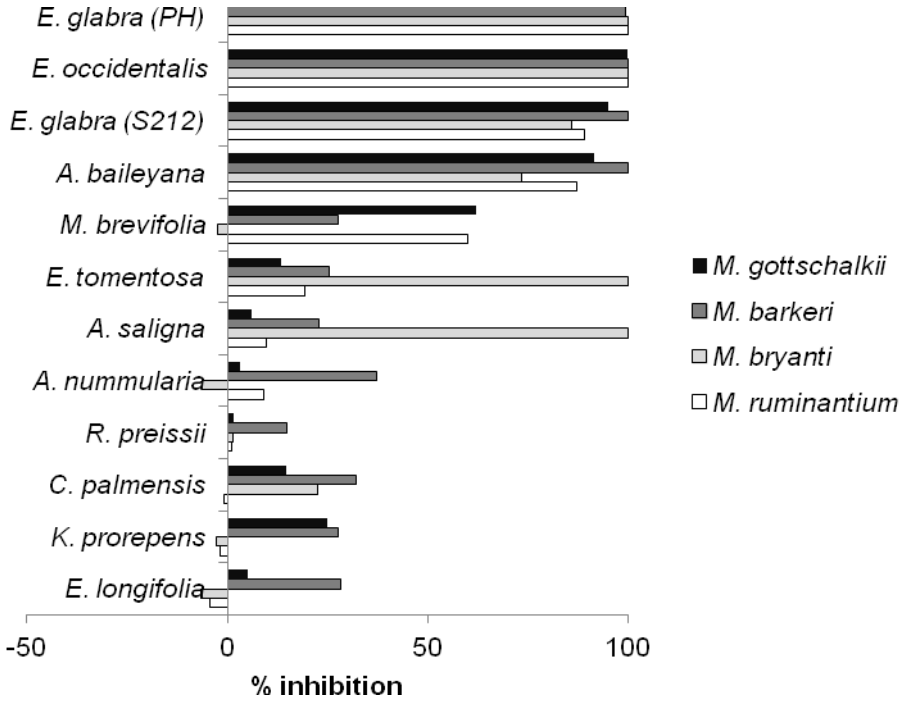

Figure 1. Inhibition of methane production by pure cultures of methanogens in presence of ethanolic extracts from selected plants when compared to cultures without extracts

In conclusion, a range of plant extracts affected selected pure cultures of methanogens. In some extracts the effect was broad (i.e. 100% inhibition of all methanogens tested), while in others partial and specific effects (limited reduction, only some methanogens affected) were observed.

Implications: Plants that can completely inhibit methanogenesis by pure cultures of rumen microbes have been identified. The broad and specific action of different plants provides evidence that there are different compounds and different mechanisms involved in the inhibition. Analysis of these effects at the chemical and molecular level may provide an insight into key differences between methanogen species that could be targeted for manipulation. The wide spectrum of activities also indicates that a combination of plants integrated into a grazing system will be effective against a range of different methanogens.

Section 4. Isolate and identify bioactive compound(s) with antimethanogenic properties from candidate plants and plant materials
(1 draft paper, 1 short discussion paper)

Draft manuscript – ASM 2012
Preliminary fractionation of anti-methanogenic compounds from *Eremophila glabra*

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Abstract
Freeze dried ground leaves of *Eremophila glabra* were extracted with different solvents to determine if they contain anti-methanogenic compounds. Analyses by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) indicated that a 1:1 ration of methanol in chloroform, yielded the highest concentrations and widest variety of organic compounds of interest. Fractions prepared by acid-base separation and column chromatography were analysed by HPLC, TLC, HPLC-Mass Spectrometry (HPLC-MS) and *in vitro* fermentation to determine compounds in *E. glabra* related to inhibition of methanogenesis by rumen microbes.

Introduction
Bioactive plants contain secondary compounds that can modulate ruminal fermentation (Wallace, 2004; Patra et al., 2006). Australian native plants from the *Eremophila* genus are known for their antimicrobial activity (Ghisalberti, 1994a;b). *Eremophila glabra* has been shown to affect methane production in the rumen (Durmic et al., 2010a) and recently we demonstrated persistent antimethanogenic effects in a continuous *in vitro* fermentation system also inhibit various types of ruminal bacteria in batch and continuous culture (Hutton et al., 2009a). Particular emphasis has been placed on the serrulatane diterpenes, as the major acidic components previously extracted from *E. glabra* (Forster 1986). However, limits in the techniques available at the time of previous studies give reason to suggest that there may be other compounds with potential for anti-methanogenic effect. Modern techniques allow for more information to be gathered about the quantities and types of compounds in *E. glabra* relating to anti-methanogensis.
Our objective was to fractionate compounds from *E. glabra* and identify those fractions that inhibit methanogenesis. Plant fractions from *E. glabra* were extracted using different solvents and analysed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC), HPLC-Mass Spectrometry (HPLC-MS). A 24 h *in vitro* batch fermentation system was used to screen the main effects of different plant fractions on ruminal fermentation.

Material and methods

The use of animals and the experimental protocol were approved by the Animal Ethics Committee of the University of Western Australia.

Plant material

Leaves were harvested from *E. glabra* plants in reproductive stage from our Monarto site in South Australia. The leaves were freeze dried and grounded prior to fractionation.

Column chromatography

Methanol in chloroform (1:1) was used to prepare extracts for column chromatography. An alumina column equilibrated with hexane was loaded with the plant extract. This was separated using various eluent systems from 100% hexane, through to 100% ethyl acetate, then to 100% methanol. The fractions were concentrated under vacuum, and dried under nitrogen. Using this procedure, eleven fractions were eluted and tested in the *in vitro* fermentation assay (Table 1).

Acid base extraction

Plant material from *E. glabra* was dissolved in chloroform, basified using $NaHCO_3$ (1M) and NaOH (0.5M), and re-acidified with HCl (4M), to separate compounds according to their acidity. The sample was separated into five fractions: a strong acids fraction, a week acids fraction 1 (using 10% chloroform), weak acid fraction 2 (using 10% methanol) and a neutral fraction (Table 2). Each fraction (equivalent to 0.5 g dry weight of plant material) was tested separately in the *in vitro* fermentability assay.

In vitro fermentation assay

Rumen fluid from two fistulated Merino sheep was collected 2 hours after morning feeding. The sheep were fed on a standard diet (a mixture of 1 part oaten chaff + 0.25 part lupin + 0.025 part minerals) *ad lib* for two weeks before sample collection. The rumen fluid was kept in pre-warmed thermos flasks, strained through 3 layers of cheesecloth, before being used as the source of inoculums.

One day before the *in vitro* fermentation assay, all plant extracts (equivalent to 0.5 g plant material) were dissolved completely in EtOH to a concentration of 0.5 g/mL and kept in sealed glass container. Prior to the incubation, oaten chaff was weighed into Bellco tubes and kept in an anaerobic chamber. The rumen fluid was adjusted to pH 7.2 with McDougall buffer (McDougall, 1948), and then 10 mL buffered rumen fluid was added to each Bellco tube. Two hundred microliters of each extract was added into the tubes afterwards. Blanks were tubes without culture substrate. Controls were oaten chaff (control 1) and oaten chaff with 0.2 mL EtOH (control 2). Each treatment was prepared in triplicate. The tubes were sealed with rubber stoppers and aluminium seals and placed in an incubator for 24 hours at 39°C with constant shaking at 50 rpm.

At the end of the incubation, gas production from the headspace of the bottle was measured using a gas meter (GMH 3110, Greisinger Electronic Gmbh, Germany). A 5 mL gas sample from the headspace of the bottle was analysed for methane concentration (using a gas chromatographer (GC, Micro GC Chromatograph, CP-4900, Varian)

Results

Column extractions

The effects of extracts of *E. glabra* extracted via column chromatography separation on total gas production, methane concentration and methane production are presented in Table 1. Total gas production was reduced by plant fractions 1, 2, 4, 9, and 10 ($P < 0.05$). Fraction 7 and 8 had the highest methane production ($P < 0.05$) and only plant fractions 1 and 9 contained antimethanogenic compounds, with reductions of 54.7% and 56.2% in methane emission, respectively ($P < 0.05$).

Acid-base extractions

All extracts obtained by acid-base the method reduced total gas and methane production by over 2% and 60% respectively, compared with the control 2 ($P < 0.05$, Table 2). Neutral extraction produced the lowest total gas and methane production compared to other treatments, with inhibition over 93% of methane emission compared to the control 2. However, weak acid fraction 2 promoted methane production by 21.6% compared to control 2 ($P < 0.05$).

Table 1. Effects of *E. glabra* fractions separated by column chromatography on ruminal fermentation (mean ± S.E.M.) in 24 h batch culture

Treatment	Total gas production (mL/g DM)	CH_4 concentration (%)	CH_4 production (mL/g)
*Control 1	361±2.87^{a}	11.4±0.48^{b}	41.3±1.47^{b}
*Control 2	357±2.88^{a}	14.1±1.06^{ab}	50.5±4.21^{b}
1	312±4.35^{f}	7.3±0.68^{c}	22.9±2.41^{c}
2	339±1.68^{bcde}	13.1±0.75^{ab}	44.3±2.76^{ab}
3	344±7.24^{abcd}	12.6±0.25^{ab}	43.2±1.16^{ab}
4	329±7.16^{edf}	11.0±1.06^{bc}	36.1±2.93^{b}
5	346±3.71^{abcd}	12.9±0.45^{ab}	44.5±1.10^{ab}
6	352±4.23^{abc}	13.3±0.36^{ab}	46.9±1.34^{ab}
7	363±5.87^{a}	15.1±0.47^{a}	54.7±2.52^{a}
8	361±0.75^{a}	15.0±0.63^{a}	54.1±2.32^{a}
9	318±1.26^{ef}	6.9±1.42^{c}	22.0±4.39^{c}
10	332±1.91^{cdef}	13.4±0.31^{ab}	44.4±1.23^{ab}
11	348±2.81^{abcd}	13.1±0.83^{ab}	45.6±3.22^{ab}

*control 1: oaten chaff ; control 2: oaten chaff with 0.2 mL EtOH

$^{a-c}$ means in a same row with different superscript letters are significantly different at $P < 0.05$.

Table 2. Effects of *E. glabra* fractions separated by acid base extraction on ruminal fermentation (mean ± S.E.M.) in 24 h batch culture

Treatment	Total gas production (mL/g DM)	CH_4 concentration in headspace (%)	CH_4 production (mL/g DM)
*Control 1	361±2.87[a]	11.4±0.48[b]	41.3±1.47[b]
*Control 2	357±2.88[a]	14.1±1.06[ab]	50.5±4.21[b]
Neutral extraction	300±3.26[c]	1.2±0.73[d]	3.5±2.26[d]
Strong acids fraction	349±3.44[b]	5.8±0.08[c]	20.2±0.16[c]
Weak acids fraction 1	352±5.30[b]	5.6±0.29[c]	18.2±0.94[c]
Weak acids fraction 2	370±2.57[a]	6.6±0.36[a]	61.4±1.64[a]

*control 1: oaten chaff; control 2: oaten chaff with 0.2 mL EtOH
[a-c] means in a same row with different superscript letters are significantly different at $P < 0.05$.

Discussion

E. glabra is a potent anti-methanogenic plant in the rumen and in this study our aim was to narrow down the search for targeted compounds with bioactivity against methanogens. Column fractions 1 and 9, and the neutral extract were most effective at reducing methane production. Since there are multiple fractions that reduce methane production, it suggests that more than one type of compound is responsible for the antimethanogenic effects of *E. glabra*. These results are a critical step in the process of purifying specific compounds responsible for the rumen modulating effects we have observed in *E. glabra*. Our expectation was that the serrulatane diterpenes would be responsible, at least in part, for the antimethanogenic effects because they are known to have antimocrobial properties (Ghisalberti 1997). The column fractions 3, 6 and 8 had high concentrations of compounds with molecular ions consistent with serrulatane diterpenes, but they did not have inhibitory effects on batch culture fermentation. We can speculate that compounds other than serrulatane diterpenes are responsible for the anti-methanogenic and other rumen modulating effects of *E. glabra* that have been observed *in vitro*. Our aim is to further purify compounds from the bioactive fractions we have identified in this study.

Our previous study suggested that *E. glabra* has the potential to be used as feed supplements to abate methane emission *in vitro*, and that this effect is closely related to the *E. glabra* concentration (dose) in the fermentation media (Li et al., 2011). Studies of the bioactive fractions we have identified in this study with pure cultures of methanogens and cellulolytic bacteria will help determine which of these extracts have a direct inhibitory effect on methanogens and which have more general effects. We also have the opportunity to identify the optimum dose and persistency of these more purified extracts on ruminal fermentation and the opportunity to study the

mechanism behind their actions. Elucidated and characterisation of the specific methane inhibitor(s) in *E. glabra* will lead to the application of *E. glabra* as a feed additive for Australian livestock.

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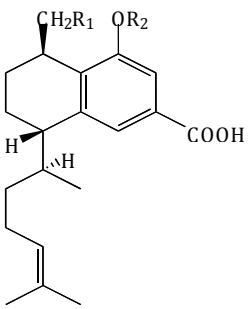
Short discussion paper - Isolation and identification of secondary compounds from Eremophila glabra

Background:

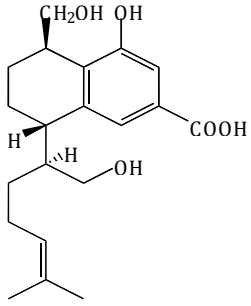
In our previous studies we have identified *E. glabra* as a potent bioactive plant against rumen methanogenesis. Ethanolic extract of this plant caused up to 50% reduction in methane production in *in vitro* batch fermentation and also inhibited 80%-100% of the growth of selected rumen methanogens when tested in pure culture (**Milestone Report April 2011). It** was also demonstrated that the effect of the extract did not diminish after adding PEG (tannin binder), implying that in this plant the antimethanogenic effect may be related to compounds other than tannins. It was also shown that methanol and acetone extracts had similar activity to ethanol extracts.

Isolation and identification of secondary compounds from Eremophila glabra

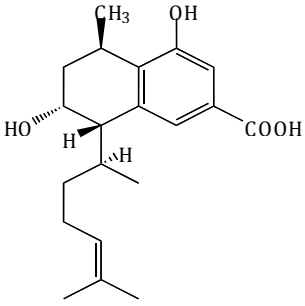
E. glabra was selected for further chemical analysis, which was conducted in collaboration with Prof Ghislaberti and Dr Flematti at UWA School of Biomedical, Biomolecular and Chemical Sciences. Briefly, the major secondary compounds were obtained from crude acetone extracts, using solvent gradients on silica gel to collect different fractions, followed by purifying fractions using High Performance Liquid Chromatography (HPLC). The fractions were separated, identified and assigned to a structure using chromatography and nuclear magnetic resonance spectroscopy. A total of seven major compounds (Compound 1 to 7) were isolated and identified as serrulatane diterpenes (Figure 1). They were similar to each other in structure, except for Compound 4 which had an additional stereocentre at C-3, and Compound 7 which lacked a double bond.



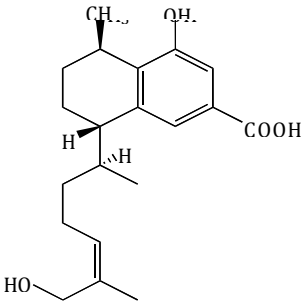
Compound 1: R_1-OAc, R_2-OAc;
Compound 2: R_1-OH, R_2-OH



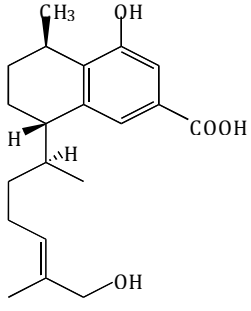
Compound 3



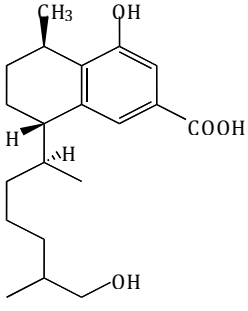
Compound 4



Compound 5



Compound 6



Compound 7

Figure 1. The structures of seven serrulatanes isolated from *Eremophila* species (G. Flematti, pers. comm.)

These compounds will now be obtained in enough quantities for bioactivity testing (*in vitro* batch fermentation). Our intention is to perform the extraction of compounds in September 2011 and test the bioactivity in October 2011 and provide the results in the final report in December.

In addition to major (non volatile) compounds, we have also investigated volatile ('essential oil-type') compounds from this plant. Compounds that were present in the plant headspace of *E. glabra* were collected and analysed using solid-phase microextraction (SPME). A range of volatile compounds, including benzene, cymene, limonene, cyclohexane etc. were identified (Figure 2). Many of these compounds are commonly found in essential oils and aromatic plants and are commercially available. If time and resources permit, some of these will be obtained and tested for bioactivity as well.

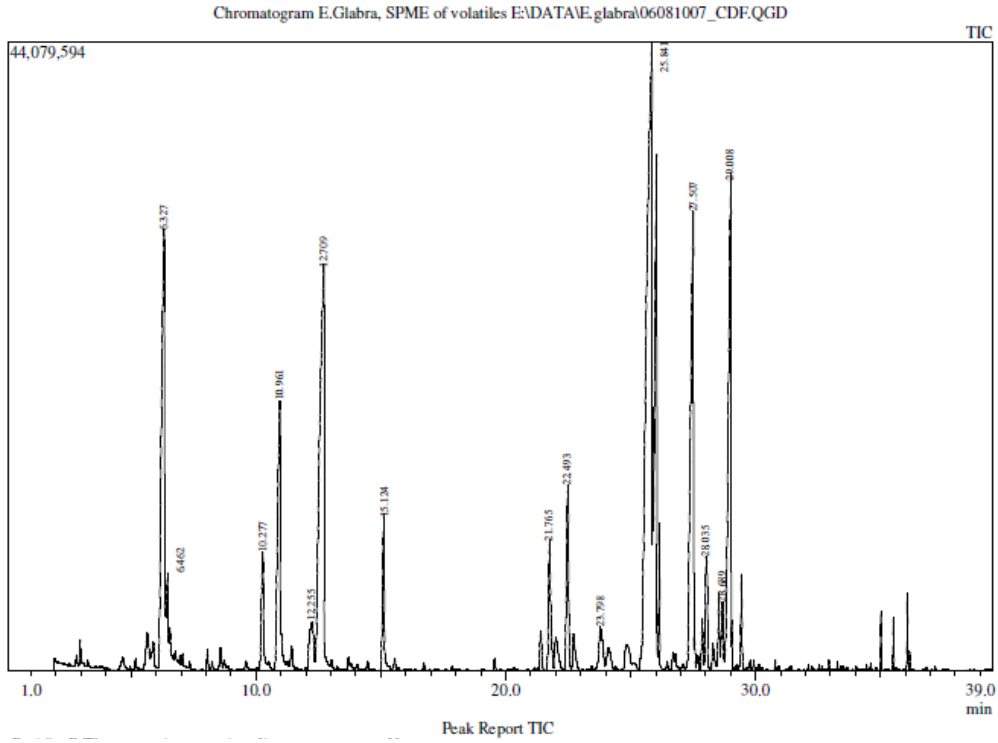


Figure 2. Chromatogram of SPME analysis of headspace of *E. glabra* (G. Flematti, pers. comm.)

Implications:

Some major secondary compounds from *E. glabra* have been isolated and identified. Once the bioactivity of these compounds is confirmed, they may serve as biomarkers for future plant screening and selection and/or development of a commercial product for methane mitigation for livestock.

Section 5. Validate *in vitro* findings *in vivo*

We will provide a draft manuscript on this Section when we have completed an animal house experiment in January/February 2012. Sheep will be fed a basal diet with 15% *E. glabra* over a period of 7 weeks. Their methane production will be measured twice during this period using respiration chambers and compared to sheep fed the basal diet only. The digestibility of the diet will also be compared and blood samples will be taken to measure blood parameters through a collaboration with C.A. Ramírez-Restrepo, leader of BCCHXXXX in RELRP.

Section 6. Papers published from BCCH1012

Bickell, S.L., Durmic, Z., Blache, D., Vercoe, P.E., Martin, G.B., 2010. Rethinking the management of health and reproduction in small ruminants. In: Updates on ruminant production and medicine, In: Eds.: F. Wittwer, R.C., H. Contreras, C. Gallo, J Kruze, F. Lanuza, C. Letelier, G. Monti & M Noro (Ed.), Proceedings of the 26th World Buiatrics Congress, [Andros Impresoroes: Chile], Santiago, Chile,, pp. pp. 317-325.

Durmic, Z., Vercoe, P., Raphalen, C., 2010. Australian native plant *Eremophila glabra* affects fermentability and reduces methane output from a sheep diet fermented in artificial rumen (Rusitec). Greenhouse Gases And Animal Agriculture Conference, October 3-8, 2010, Banff, Canada T55, 90.

Durmic, Z., Hutton, P., Revell, D.K., Emms, J., Hughes, S. and Vercoe, P.E. (2010). In vitro fermentative traits of Australian woody perennial plant species that may be considered as potential sources of feed for grazing ruminants. Animal Feed Science and Technology 160, 98-109.

Durmic, Z., Vercoe, P., Revell, D., 2009b. Bioactive plants for livestock production. Evergreen Farming December, 12-13.

Li, X.X., Durmic, Z., Liu, S.M., Vercoe, P.E., 2010. Eremophila glabra and Kennedia prorepens Reduces Methane Emission from Medicago sativa In Vitro. Proc. Aust. Soc. Anim. Prod. 28, 67.

Li, X.X., Durmic, Z., Liu, S.M., Vercoe, P.E., 2011. Dose-dependent additions of Eremophila glabra reduce methane production in RUSITEC. Advances in Animal Biosciences: Proceedings of the Eighth International Symposium on the Nutrition of Herbivores (ISNH8), pp.562.

Martin, G.B., Durmic, Z., Kenyon, P.R., Vercoe, P.E., 2010. Clean, green and ethical' animal reproduction: extension to sheep and dairy systems in New Zealand. Proceedings of the New Zealand Society of Animal Production 69: 140-147.

Ramírez–Restrepo, C.A., Xixi, L., Durmic, Z., Vercoe, P., Gardiner, C., O'Neill, C., Coates, D., Charmley, E., 2011. Assessment of tropical legumes to reduce methane emissions from pastoral systems using in vitro fermentation and near infrared reflectance spectroscopy methodologies. Greenhouse conference 4–8 April, Cairns, Australia.

Section 7. Collaboration with other projects in the Reducing Emissions from Livestock Program

The main collaboration we have had with other projects within the Reducing Emissions from Livestock Program has been with:

BCCH 1004 & 1009 (Farming systems research) in collaboration with Dr Ramirez, Dr Charmley and Dr Tomkins through the exchange of plant material, data and preparing a manuscript on screening of NorthQLD pasture species. We will also be collecting blood samples from the in vivo experiment for analysis by Dr Ramirez.

BCCH 1010 & 1014 (Mitigants & Bioactives)
In collaboration with Dr Moate and the exchange of plant material from novel forages, feed additives, industry by-products and dietary additives, as well as data, planning experimental designs and preparation of a manuscript on screening of novel forages, industry by-products and dietary additives.

BCCH 1031 (Ridgefield demonstration site)
E. glabra was planted, grown and harvested to supply the *E. glabra* needed for our *in vivo* (chamber) study in January/February 2012. AEC approval for in vivo experiment obtained and plant material has been harvested and dried. We have interacted with the RELRP team as a whole at all technical meetings and workshops held since the inception of the programme.

B.CCH. 1008 (Microbial profiling, Dr Valeria Torok, SARDI)
The group communicated and provided rumen fluid samples to Dr Valeria Torok (SARDI) to help establish methodology for testing microbial profiles in rumen fluid collected from the animals in methane emission trials. The two research groups also agreed to provide rumen fluid samples from the *in vivo* experiment at UWA/CSIRO that we have designed to test the antimethanogenic potential of *E. glabra*. The parties agreed on sampling procedures, schedules, testing and potential publications arising from the findings.

Section 8. Media coverage

List of radio interviews:
ABC Goldfields WA (Perth), 06:30 News, 26/02/2009 06:33AM
ABC Midwest Wheatbelt (Geraldton), 07:30 News, 26/02/2009 07:33AM

Television:

ABC Landline - Home Grown Fodder, Published: 22/08/2010, Reporter: Sean Murphy

List of newsletter, newspaper articles and web listings:

- Countryman, Natives may hold solution, 25-Aug-2011, pg 11
- Lachlander – Condoblin, Forage shrubs – a valuable part of the feedbase puzzle, 15-Mar-2011, pg 4
- Eyre Peninsula Tribune,Enrich your farms with fodder shrubs, 24-Mar-2011, pg 21

- Hopetoun Courier & Mallee Pioneer, Walpeup to host forum on alternative fodder options, 31-Mar-2011, pg 2
- North West Express, Alternative fodder forum at Walpeup, 31-Mar-2011, pg 5
- Central Midlands & Coastal Advocate, Moora carbon farming workshop, 07-Apr-2011, pg 10
- Stock Journal, Shrub mix enriches outcomes, 14-Apr-2011, pg 35
- Countryman, Don't shrug off shrubs, 28-Apr-2011, pg 52
- Countryman, Scientists on emissions, 21-Apr-2011, pg 14
- Central Midlands & Coastal Advocate, Moora carbon farming workshop, 28-Apr-2011, pg 12
- Fatcow (2011), Title: UWA on Reducing Controversial Agricultural Green House Gas Emissions (Link: http://www.fatcow.com.au/c/Fatcow-www-fatcow-com-au/UWA-on-Reducing-Controversial-Agricultural-Green-House-Gas-Emissions-n913378)
- ScienceNetwork **(**2011), Title: Tar bush sheep supplement alludes to reduced methane emissions (Link: http://www.sciencewa.net.au/3647.html)
- UWA university news (2011), Title: A touch of the tar bush (Link: http://www.news.uwa.edu.au/ioa/research/touch-tar-bush)
- Subiaco Post, 17/Sep/2011, pg 26 -Fewer farts in the paddock
- The Land, 15/Sep/2011, pg 25 - Ag emissions
- Countryman, 22/Sep/2011, pg 55 - Methane control goes native
- Feedback magazine (March 2010) – Smart plants the key to methane production
- UWA News (October 2009, Vol. 28, Number 16) - Burpless sheep:a bonus for farmers and the environment (pdf attached)
- Macleay Argus, Kempsey, NSW (November 17, 2009) – Native plants can aid stock health
- UWA News (October 2009, Vol. 28, Number 16) - Burpless sheep:a bonus for farmers and the environment (pdf attached)
- Future Farm Industries CRC media release October 2009 – pdf provided
- Farm Weekly (Dec 22, 2009) – Climate no laughing matter at UWA (http://fw.farmonline.com.au/news/state/agribusiness-and-general/general/climate-no-laughing-matter-at-uwa/1708121.aspx)
- "Get Farming Australia" - Native plant chemistry could play an important role in improving livestock health (http://www.getfarming.com.au/pages/farming/articles_view.php?fId=9200020091019212831)

Producer, field, open days:
UWA Open Day, 2009, 2010, 2011
CCRP Progress Meeting, Sydney, August, 2010
Dowerin Field Day, August 2010 and 2011
UWA Future Farm, October 2010 and 2011
CCRP Producer Forum, Canberra, March, 2011
UWA Special display in UWA Museum, June-July 2011
Enrich Road show March-April 2011, NSW, SA, WA