Eremophila glabra reduces methane production in sheep

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Summary

Ruminant methane (CH_4) production is the major source of agriculture CH_4 emission in Australia and mitigating its emission is a priority. Substituting bioactive plants to the diet can alter rumen fermentation and reduce enteric CH_4 production. *Eremophlia glabra* Juss. (Scrophulariaceae), a native shrub, reduces CH_4 production but also has general inhibitory effect on fermentation when used as the sole substrate *in vitro*. This general effect can be reduced by mixing *E. glabra* with other substrates. *E. glabra* is fully adapted to the Australian Mediterranean climate and has potential to be used as an alternative feed source for livestock during the 'feed gap' in summer and autumn. This thesis explores the use of *E. glabra* as one component of a mixed diet to achieve low CH_4 production without reducing animal production in sheep. My research sought to (i) evaluate the potential of *E. glabra* as a natural CH_4 inhibitor, (ii) assess the feasibility of using *E. glabra* in the diet of sheep, (iii) investigate the mechanisms of action behind the antimethanogenic bioactivity, and (iv) determine the effective fractions and compounds in *E. glabra* responsible for the antimethanogenic effects. In this thesis, I initially tested the antimethanogenic and general antimicrobial effects of *E. glabra* in short-term (24 h) and long-term (3 weeks) *in vitro* fermentation and subsequently *in vivo*. I then explored the components of *E. glabra* responsible for the bioactivity.

The *in vitro* studies were used to assess a range of substitutions of *E. glabra* with oaten chaff/ lupin substrate and to select an effective level that reduced CH_4 production with minimal or no general effects on overall fermentation. The *in vitro* work was undertaken in two stages: 24 h batch culture fermentation (Chapter 3) and a continuous fermentation (Rusitec, Chapter 4). Seven levels of *E. glabra* were substituted with oaten chaff /lupin and compared in batch fermentation (Chapter 3). *E. glabra* substitutions decreased production of total gas and CH_4 in a dose-dependent manner without affecting the concentration of total volatile fatty acids. Overall, at a substitution rate of less than 50%, *E. glabra* can reduce CH_4 production with minimal effects on overall rumen fermentation.

Based on the batch fermentation results, three levels of *E. glabra* (EG15: 15%, EG25: 25% and EG40: 40%) were fermented in a Rusitec (continuous *in vitro* fermentation system) for three weeks to examine their overall effects on fermentation and microbial ecology, as well as the persistency of their effects (Chapter 4). *E. glabra* substitutions

decreased the production of total gas, CH_4 and volatile fatty acids in a dose-dependent manner. All *E. glabra* substitutions decreased the methanogen population and had differential effects on numbers of cellulolytic bacteria. Overall, substituting 15% of *E. glabra* was the most promising combination that reduced the methanogen population and CH_4 production over three-week with some minor inhibition of overall fermentation.

Following the Rusitec results, an *in vivo* experiment was conducted on one-year old wethers (Merino×Suffolk) fed 15% *E. glabra* substituted with oaten chaff/lupin for one-month to confirm its ability to mitigate CH_4 (Chapter 5). Compared to sheep fed the control diet (oaten chaff/ lupin), sheep fed the 15% *E. glabra* diet reduced CH_4/digestible dry matter by 14.8%. The *E.* glabra diet did not affect rumen fermentation, feed digestibility or animal health. These findings show that *E. glabra* could be fed to sheep as a minor component of the diet to reduce CH_4 emission, while maintaining animal productivity.

In the final experimental chapter (Chapter 6), a stepwise approach was used to fractionate extracts of *E. glabra* that contained antimethanogenic activity. The *in vitro* batch fermentation system was used as the bioassay for detecting bioactivity of extracts and different fractions. Initial crude extracts were fractionated using silica gel column chromatography and the active fractions generated at this stage were separated further by high performance liquid chromatography (HPLC). However, none of the fractions from the HPLC isolation reduced CH_4 production. This study demonstrate that it is possible to achieve some level of purification of extracts from *E. glabra* that can reduce CH_4 production, but the identity of the specific compounds responsible are yet to be elucidated.

Collectively, this research demonstrates that the native shrub, *E. glabra*, can mitigate CH_4 production and can be used as forage for sheep. I have identified a target level of *E. glabra* that would need to be consumed in a grazing system to reduce CH_4 emissions and contribute to productivity. This project has provided a systematic evaluation procedure that can be applied to other novel plants and emphasises the importance of transferring results from *in vitro* batch and continuous fermentation systems to *in vivo*. The specific compounds responsible for the antimethanogenic bioactivity in *E. glabra* have not been identified yet, but there is preliminary evidence that it will be feasible to do so. Identifying these compounds will enable the mechanisms behind the bioactivity to be investigated.

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Statement of contribution

The research presented in this thesis is an original contribution to the mitigation of ruminant methane using *Eremophila glabra*, an Australian native plant with bioactive properties.

This thesis is presented as a series of independent research papers (to be published), preceded by a general introduction chapter and followed by general discussion. The experimental chapters of this thesis are written and presented as separate manuscripts, so some repetition of basic information does occur.

The experimental design and manuscript preparation were carried out by me after discussion with and review by my supervisors, Dr Phil Vercoe, Dr Shimin Liu and Dr Zoey Durmic. This thesis does not incorporate any material previously submitted for a degree or diploma at this or any other university or institute, and it does not contain any material previously published or written by another person. The publications on the following page have been co-authored by my supervisors.

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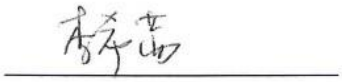
Shimin Liu

Zoey Durmic



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List of abbreviations

A:P	Acetate:propionate ratio
ADF	Acid detergent fibre
AFIA	Australian fodder industry association
CH_4	Methane
CT	Condensed tannins
DM	Dry matter
Dmi	Dry matter incubated
EO	Essential oil
EtOAc	Ethyl acetate
EtOH	Ethanol
F	ANOVA statistics
GC	Gas chromatography
HPLC	High performance liquid chromatography
LSD	Least significant difference
MeOH	Methanol
MS	Mass spectrometry
NDF	Neutral detergent fibre
NH_3	Ammonia
PEG	Polyethylene glycol
Rusitec	Continuous *in vitro* fermentation
UWA	The University of Western Australia
VFA	Volatile fatty acids
α	Alpha (significance level)

Publications arising from this thesis

Refereed publication (accepted by Anaerobe, pending minor revision):

Li, X., Durmic, Z., Liu, S.M., McSweeney, C. & Vercoe, P.E. 2013, '*Eremophila glabra* reduces methane production and methanogen populations when fermented in a Rusitec'. *Anaerobe,* http://dx.doi.org/10.1016/j.anaerobe.2013.10.008. (Chapter 4).

Conference paper

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Li, X., Raphalen, C., Durmic, Z., Liu, S.M. & Vercoe, P.E. 2012, 'Supplementation of substrate with *Eremophila glabra* reduces methane production in *in vitro* fermentation', *Proceedings of the 15th Asian-Australasian Association of Animal Production Societies (AAAP) Animal Science Congress*, Bangkok, Thailand, p. 86. (Part of Chapter 3)

Li, X., Liu, S.M., Durmic, Z. & Vercoe, P.E 2011, 'Dose-dependent additions of *Eremophila glabra* reduce methane production in RUSITEC', in *the Eighth International Symposium on the Nutrition of Herbivores (ISNH8),* Advances in Animal Bioscience, Wales, UK, p. 562. (Part of Chapter 4)

Restrepo, C.R., **Li, X.**, Durmic Z., Vercoe, P., Gardiner C., O'Neill, C., Coates, D., Charmley, E. (2011). *In vitro* assessment of the antimethanogenic potential of tropical legumes to reduce methane emissions from northern grazing systems. *Greenhouse*, Cairns Convention Centre, Queensland, Australia, p.87. (Pre-chater 3 experiment)

Li, X., Durmic, Z., Liu, S.M. & Vercoe, P.E. 2010, '*Eremophila glabra* and *Kennedia prorepens* reduces methane emission from *Medicago sativa in vitro*', in *28th Australian Society of Animal Production*, Armidale, NSW, Australia, p. 67. (Pre-Chapter 3 experiment)

Chapter 1 General Introduction

Methane (CH_4) is the second largest greenhouse gas after CO_2, and is 25 times more efficient than CO_2 at trapping heat in the atmosphere (Forster et al. 2007). Global agriculture contributes approximately 47% of anthropogenic CH_4 emissions (Smith et al. 2007). Agricultural CH_4 emission arises primarily from ruminant livestock with cattle and sheep, accounting for 25% to 44% of anthropogenic CH_4 emissions (Denman & Brasseur 2007; Smith et al. 2007; Rodr ǵuez-Romero et al. 2013). It has been estimated that the combined CH_4 emissions from enteric fermentation and manure management will increase by 21% between 2005 and 2020 (Smith et al. 2007). In Australia, ruminants are the single largest source of agricultural CH_4 emissions, contributing 65% of agriculture greenhouse gas, which is approximately 9.3% of the total greenhouse gas emissions (National Greenhouse Gas Inventory 2011). Consequently, CH_4 emissions from ruminants are causing concern in Australia and mitigating their emissions is a priority.

In ruminant, CH_4 is produced by methanogenic Archaea (methanogens) that utilize the hydrogen and carbon dioxide produced during fermentation of feed in the rumen. By producing CH_4, methanogens play a role in preventing the accumulation of hydrogen in the rumen, which is toxic to the ruminal microorganisms. However, CH_4 production can represent an energy loss of up to 15% of gross energy from ruminants depending on the type of diet being fed (Johnson & Johnson 1995; McAllister et al. 1996; Van Nevel & Demeyer 1996). Manipulating the diet is one of the most promising ways to reduce enteric CH_4 production (Johnson & Johnson 1995; McCaughey et al. 1999; Benchaar et al. 2001; Aguerre et al. 2011) because it offers the potential to influence rumen fermentation to : 1) reduce hydrogen production without adversely affecting feed efficiency; 2) alter the pathways of utilization of hydrogen and 3) reduce both the number and the activity of methanogens (Martin et al. 2010). Importantly, any attempt to reduce CH_4 emissions from ruminants by manipulating the diet should not be at the expense of animal performance.

The diet can be manipulated by including plants containing secondary compounds that influence microbial fermentation in the rumen and reduce CH_4 production *in vitro* (Soliva et al. 2008; Patra & Saxena 2010; Durmic & Blache 2012). South Western Australia is recognized as one of the global hot spots for biodiversity (Mittermeier &

Russell 2004) and the native plants in this region are wild and rich in plant secondary compounds. There is growing interest in integrating Australian native perennial shrubs into pasture systems in the semi-arid grazing regions of Australia, because they are adapted to the Australian climate and soil types and offer production and natural resource management benefits (Revell et al. 2008; Moore et al. 2009). There is evidence that some of the native shrubs contain bioactive secondary compounds and one of particular interest is *Eremophlia glabra* Juss. (Scrophulariaceae). *E. glabra* has been demonstrated to have general antimicrobial properties (Ndi et al. 2007), antimethanogenic (Durmic et al. 2010a; Durmic et al. 2013) and anthelmintic properties (Kotze et al. 2009), and the potential to prevent lactic acidosis in sheep (Hutton et al. 2010; Durmic et al. 2012). However, *E. glabra* also has a general inhibitory effect on fermentation when it is used as the sole substrate *in vitro* (Durmic et al. 2010a), which is undesirable because it suggests that it inhibits more than just the microorganisms involved in the pathways of CH_4 production.

Bioactive plants can have dose dependant effects on rumen fermentation when mixed with other substrates (Castillejos et al. 2007; Garc á-Gonz áez et al. 2008b; Goiri et al. 2009; Zhang et al. 2011). The antimethanogenic effects of *E. glabra* are strong and it is possible that using small amounts in a mixed substrate may moderate the more general antimicrobial effects on fermentation, without eliminating entirely the beneficial antimethanogenic effects. In this thesis I have explored whether this is possible. The general hypothesis tested in this thesis was that *E. glabra* could be fed to sheep as part of a mixed diet to reduce CH_4 production without causing a reduction in production parameters. To test this hypothesis, I compared different levels of *E. glabra* in *in vitro* batch and continuous (Rusitec) fermentation systems and measured fermentation parameters (eg. production of total gas, CH_4, concentration of volatile fatty acids and ammonia) to establish a level that reduced CH_4 production but did not affect overall fermentation. This information was used to select a level of *E. glabra* to feed sheep in an animal house experiment to verify its effects *in vivo*. I also attempted to extract and isolate the bioactive components responsible for the antimethanogenic effects of *E. glabra.*

Chapter 2 Literature review

Opportunities to mitigate ruminant methane using bioactive plants

2.1 Introduction

The concentration of greenhouse gases in the atmosphere has risen with the increase in human population and human activities (Forster et al. 2007). Methane (CH_4) is the second most important gas involved in global warming and accounts for 14% of the human-induced production of greenhouse gases (Forster et al. 2007). It has a global warming potential of 25 times higher than CO_2 (Forster et al. 2007). The sources of CH_4 emissions are usually divided into two types: natural emissions and anthropogenic emissions – human induced. The focus of this review is on the anthropogenic CH_4 emissions specifically related to agriculture and enteric emissions from ruminants.

Table2.1 Estimated annual enteric CH_4 emissions from the main domesticated livestock species (Sauvant 1992).

	CH_4 emission (g $kgBW^{-1}$ $year^{-1}$)	Assumed average bodyweight (kg)	CH_4 emissions (kg $animal^{-1}$ $year^{-1}$)
Ruminants			
Dairy cows	150	600	90
Beef cattle	163	400	65
Sheep	160	50	8
Goats	160	50	8
Non-ruminants			
Swine	13	80	1
Poultry	-	2	<0.1
Horses	30	600	18

Agricultural activities are responsible for about 47% of anthropogenic CH_4 emissions with enteric CH_4 from ruminants, mainly from dairy cows, beef, cattle and sheep representing the largest contributions (86 million tonnes or 25 to 44% of anthropogenic CH_4 emissions; (Denman & Brasseur 2007). Approximately, 18.9 million tonnes of CH_4 are from dairy cattle, 55.9 million tonnes from beef cattle, and 9.5 million tonnes from sheep (Table 2.1,McMichael et al. (2007)). In Australia, agriculture contributes approximately 14% of the national greenhouse gas emissions (National Greenhouse Gas Inventory 2011). CH_4 production from enteric fermentation in ruminants accounts for 65% of the agricultural greenhouse gas emissions, which is approximately 9% of the Australian greenhouse gas emissions. This amount of CH_4 is expected to increase in coming decades due to the rising demands for animal products and this has led to a

growing interest in mitigating CH_4 emissions from ruminants without affecting their productivity. Since CH_4 production is an energy loss of fermentation in the rumen, reducing CH_4 emissions will also improve the efficiency of feed utilisation making the quest to reduce CH_4 emissions without reducing productivity possible.

The main strategies being considered for reducing CH_4 emissions include improving feed quality, feed additives and diet substitution (chemicals additives, bioactive plants and plant extracts) and genetic selection (Moss et al. 2000; Beauchemin et al. 2008; Hart et al. 2008; Eckard et al. 2010; Bodas et al. 2012). Dietary management is most likely to make an immediate impact on mitigating CH_4 because it targets the source of the CH_4 emissions, the ruminal microbial population, directly. CH_4 is formed by methanogenic Archaea (methanogens) that utilize the hydrogen and carbon dioxide (CO_2) produced during fermentation of feed in the rumen. By producing CH_4, methanogens play a role in preventing the accumulation of hydrogen in the rumen, which is toxic to the ruminal microorganisms. Manipulating the diet can have a direct effect on the structure of the rumen microbial population, divert hydrogen away from CH_4 production or inhibit methanogenesis and, therefore, reduce CH_4 emission per unit of feed digested in ruminants (Johnson & Johnson 1995; Benchaar et al. 2001; Beauchemin et al. 2008; Martin et al. 2010).

This review addresses the potential use of bioactive plants for CH_4 abatement and identifies research and development opportunities for making use of Australian native plants, but the scope is limited to one particular species - *Eremophila glabra* Juss. (Scrophulariaceae). It will also address the possibility of incorporating native shrubs into farming systems to reduce methanogenesis from ruminants because they have been adapted to Australian Mediterranean climate and can be alternative feed sources for livestock during the feed gap in summer and autumn. There are three main aspects of CH_4 production from ruminants covered in the review: 1) CH_4 formation in the rumen; 2) use of bioactive plants and extracts to mitigate ruminant CH_4 production, and; 3) the potential use of *E. glabra* in mitigating CH_4 emission. There is also a need to cover the value of *in vitro* methods to screen for potential CH_4 inhibitors as well as the need to then test them in the *in vivo*. An overarching hypothesis is proposed based on the review of literatures.

2.2 CH_4 formation - modulating hydrogen in the rumen will alter CH_4 emission

2.2.1 CH_4 formation and hydrogen balance

Ruminants are distinguished by their highly specialized, compartmentalized stomach system. The largest of them is known as the rumen, where a symbiotic population of microbes secrete enzymes that allow breakdown of plant cell walls and other ingested components by the host ruminant. The breakdown process is conducted mainly by cellulolytic bacteria such as *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*, which are dominant species in the rumen (Sinha & Ranganathan 1983; Forsberg et al. 1997; Krause et al. 2003). This fermentation yields energy for the microbes and the resulting fermentation end-products, mainly volatile fatty acids (VFA), are absorbed by ruminant and used as its main energy source. The microbial biomass forms a valuable source of protein to the ruminant as it flows out of the rumen to be digested in the small intestine. During the fermentation process, CH_4 is formed as a by-product and is exhaled and eructed by the host animal (Murray et al. 1976). As CH_4 is exhaled and belched into the atmosphere, the ruminant suffers a loss of ingested food-derived energy by 2-15% depending upon the diet (Johnson & Johnson 1995; McAllister et al. 1996; Van Nevel & Demeyer 1996). Reducing CH_4 emissions, therefore, offers the dual benefits of reducing greenhouse gas emissions and improving feed efficiency.

During fermentation of feedstuffs in the rumen, CH_4 is produced by a very specific group of microorganisms, called methanogens through a process known as methanogenesis. Methanogens, a distinct group of single-celled microorganisms, belong to the domain Archaea and the phylum Euryarchaeota (Woese et al. 1990; Pace 2006). All methanogens contain coenzyme $F_{420,}$ which is a cofactor necessary for enzymes such as hydrogenase and formate dehydrogenase. Another characteristic coenzyme is coenzyme M, which is either produced by the methanogens such as *Methanobacterium*, or is required from an external source, which is the case for *Methanobrevibactor ruminantium* (Rouviere & Wolfe 1988). Coenzyme M or 2-mercaptoethanesulfonic acid, is methylated to produce CH_4 (Hobson & Stewart 2011). The three most common methanogenic reactions that occur in ruminants are as follows (Whiteman et al. 1991):

$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$

$4\text{Formate} \rightarrow CH_4 + 2H_2O + 3CO_2$

$\text{Acetate} \rightarrow CH_4 + CO_2$

CH_4 formation occurs mainly through the conversion of CO_2 to CH_4 by using H_2 (Hungate 1966; Demeyer & Van Nevel 1975). In addition, formate (IUPAC name: methanoate) – a derivative of formic acid, which is formed in the concentration of acetate, can also be used as a substrate for methanogenesis, although it is often converted quickly to H_2 and CO_2 instead (Hungate et al. 1970). Because of its rapid conversion to H_2 and CO_2, it is very hard to detect the exact amount of CH_4 being produced from formate (Garcia et al. 2000).

The amount of CH_4 production depends on hydrogen (a major end-product of ruminant microbial digestion) availability in the rumen. Managing hydrogen production and its utilization is considered the key factor in controlling CH_4 emissions in ruminants (Joblin 1999; Janssen 2010). A pathway of rumen fermentation related to metabolic hydrogen production and its utilization is presented in Figure 2.1. Hydrogen is generated mostly from the breakdown of the carbohydrates in plant cell walls to acetate and butyrate by the cellulolytic bacteria such as *R. albus* and *R. flavefaciens* (Singh & Jain 1986; Moss et al. 2000). The acetate that is produced during fermentation can be used as an indicator of hydrogen production in the rumen. Hydrogen utilisers, on the other hand, provide pathways to dispose the hydrogen accumulated in the rumen, which is toxic to ruminal microbes. Methanogen and propionate producers are two known hydrogen utilisers. It is considered critical to dispose H_2 quickly and efficiently in order to maintain a vigorous fermentation. This role is mainly accomplished by methanogens (Wolin 1960).

The types of VFA formed in the rumen are also associated with CH_4 formation, because acetate and butyrate represent hydrogen sources (Wolin & Miller 1982), while propionate competes for hydrogen with the methanogens (Wolin 1960; Van Nevel et al. 1974; Van Nevel & Demeyer 1996). The acetate to propionate ratio (A:P) is highly positively correlated with CH_4 production (Russell 1998; Janssen 2010). Therefore, promoting propionate production, which requires hydrogen, can reduce CH_4 production.

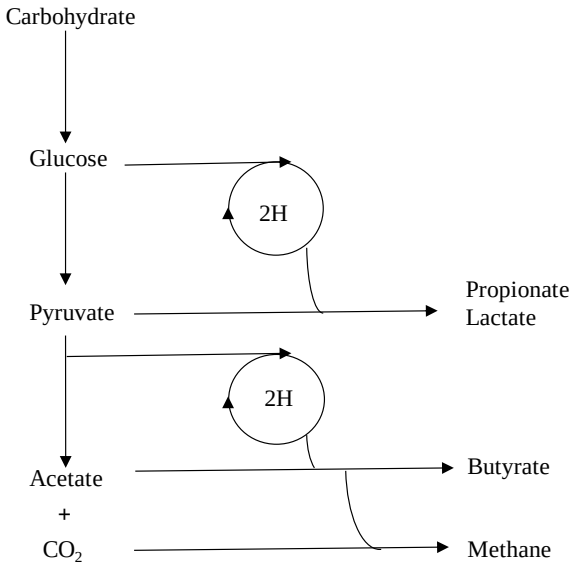

Figure 2.1. A pathway modified from Demeyer and De Graeve (1991) of rumen fermentation of carbohydrate with production and utilization of metabolic hydrogen (H).

2.3 Dietary mitigation strategies from ruminants

Dietary manipulation aims to increase feed digestibility, promoting propionate production and reducing methanogenesis. Dietary mitigating strategies fall into two categories: 1) improving feed quality and 2) dietary substitutions such as feed additives, bioactive plants and plant extracts (Beauchemin et al. 2008; Eckard et al. 2010; Mart ńez et al. 2010b; Patra & Saxena 2010; Flachowsky & Lebzien 2012; Clark 2013). This section focuses mainly on using bioactive plants and plant extracts to mitigate ruminant CH_4 production.

2.3.1 Improving feed quality

The feed components, especially the type of carbohydrate, are important for CH_4 production in ruminants as they are able to influence the ruminal pH and subsequently alter the microbiota present (Johnson & Johnson 1995; Mitsumori & Sun 2008; Hook et al. 2010; Szumacher-Strabel & Cieslak 2012). The fermentation of starch and other non-structural carbohydrates favours propionate production and thereby reduces CH_4 emission. For example, using a legume rather than grass forage can lead to low CH_4 production (Demeyer & Van Nevel 1975; Moss et al. 2000; Benchaar et al. 2001; Boadi et al. 2004). The quality of forage affects the activity of rumen microbes and CH_4 production in the rumen. Forage species, forage processing, the proportion of forage in diet and the source of the grain also influence CH_4 production in ruminants. CH_4

production tends to decrease as the protein content of feed increases, and increases as the fibre content of feed increases (Sekine et al. 1986; Shibata et al. 1992; Johnson & Johnson 1995; Kurihara et al. 1997). The digestibility of cellulose and hemicelluloses are strongly related to CH_4 production, more so than soluble carbohydrate (Holter & Young 1992). High proportion of digestibility of hemicelluloses and low proportion of digestibility of cellulose in the diet is related to low CH_4 output in non-lactating cows fed forage (Holter & Young 1992). The relationship between CH_4 production and proportion of concentrate in the diet is curvilinear: when the proportion of concentrate in the diet increases, the CH_4 production decreases, thereafter, CH_4 production stays constant at 30–40% concentrate levels in the diet and then increases with a concentrate proportion of 80–90% (Sauvant & Giger-Reverdin 2007). However, increasing the amount of rapidly fermentable carbohydrates in a diet can increase the production of VFA and reduce the CH_4 formation in the rumen, as well as lower the ruminal pH (Eadie et al. 1970). This may further inhibit methanogens and other rumen ciliates (Eadie et al. 1970; Esdale & Satter 1972; Van Kessel & Russell 1996). If VFA production is greater than their absorption across the rumen epithelium, it can cause hydrogen accumulation in the rumen, which decreases the pH, leading to ruminal acidosis and disruption of rumen fermentation (Plaizier et al. 2008). It is important to note that CH_4 reduction should not compromise rumen fermentation. It has been suggested that increasing feed quality is an efficient strategy to improve animal performance and reduce hydrogen available for methanogens, and thereby, lowering CH_4 emission per unit of animal product.

2.3.2 Bioactive plants for reducing CH_4 emission

Chemical additives can be used as dietary additives to modulate rumen fermentation. Various chemical additives have been studied to manipulate rumen fermentation (Erdman 1988; Russell & Chow 1993; Clark et al. 2009). For example, 2-bromoethanesulphonate can reduce propionate concentration and the total population of methanogens (Zhou et al. 2011). Bromochloromethane has a persistent effect on reducing CH_4 for 9 days in continuous fermentation and can inhibit the population of methanogens and *F. succinogenes* (Goel et al. 2009). Monensin – a polyether antibiotic isolated from *Streptomyces cinnamonensis* has been widely used in cattle for improving feed efficiency (Turner et al. 1977; Van Baale et al. 2004). It has been reported that monensin can have long term effects up to 47 days in modulating VFA production (Ellis

et al. 2012b). Monensin can also decrease protozoa numbers in the rumen of sheep for up to 28 weeks (Habib & Leng 1986). Moreover, there is a concern amongst consumers that rumen microbes may develop resistance to these antimicrobial additives, and the resistance may be passed on from livestock to human pathogens (Alexander et al. 2008). This concern has lead to the prohibition of using antibiotics as growth promotants, including monensin, by the European Union in animal feed (European Parliament 2003).

Because of the risk associated with chemical additives, there is a growing interest in the use of bioactive plants and their extracts to manipulate rumen fermentation (Calsamiglia et al. 2007; Garc á-Gonz ález et al. 2008a). Plants with antimicrobial properties, called 'phytochemicals' may be used to reduce CH_4 emission (Wallace et al. 2002). To date, hundreds of plant species have been screened worldwide to assess their potential for modifying rumen fermentation *in vitro*, in particular, their effectiveness for decreasing CH_4 production (Patra et al. 2006; Bodas et al. 2008; Garc á-Gonz ález et al. 2008a; Kamra et al. 2008; Soliva et al. 2008; Durmic et al. 2010a). The European Union collaborative project, Rumen-up (EC Project, QLK5-CT-2001-00992) screened 450 plant species for antimethanogenic activity in the *in vitro* fermentation. A total of 35 plant species were found to decrease CH_4 production *in vitro* by more than 15%, and 6 plant species by more than 25% (Bodas et al. 2008). The Australian Enrich project evaluated 128 Australian native shrubs and found that more than 25% of those plant species reduced CH_4 *in vitro* in the range of 25-90% (Durmic et al. 2010a). Plants such as *Rheum officinale* and *Frangula alnus* (Garc á-Gonz ález et al. 2008b), *Eremophlia glabra* (Durmic et al. 2010a), *Rheum nobile* and *Salix caprea* (Bodas et al. 2008), *Albizia rhizonse* and *Sapindus saponaria* (Soliva et al. 2008), *Terminalia chebula* (Patra et al. 2006), have been tested for their potential to modify rumen fermentation. These bioactive plants and their extracts could be used either directly or indirectly to inhibit methanogens and their effects are most likely due to phytochemicals that alter microbial function and fermentation in the rumen (Busquet et al. 2006; Patra et al. 2006; Kamra et al. 2008; Geraci et al. 2012; Goel & Makkar 2012; Cieslak et al. 2013). While bioactive plants and plant bioactive compounds can reduce methanogenesis, they have also been reported to have side effects such as reducing VFA production, protein degradation and numbers of cellulolytic bacteria (Table 2.2, Hassanat & Benchaar (2013)). Research in this area has focused on how to minimize the potential negative effects while substituting bioactive plants and/or their extracts into an animal's diet in order to

achieve low CH_4 output. Studies on bioactive plants and their extracts are discussed below.

2.3.2.1 CH_4 mitigation using bioactive plants

Bioactive plants are rich in plant secondary compounds (Frazier 1965; Ghisalberti 1994a), which can modulate rumen fermentation and mitigate CH_4 production (Wallace et al. 2002; Bodas et al. 2008). Plants, such as rhubarb (*Rheum officinale* L.), alder Buckthorn (*Frangula alnus* L.), garlic (*Allium sativum* L.), have been shown to decrease CH_4 production, A:P and total gas production *in vitro* (Garc á-Gonz áez et al. 2008a). When bioactive plants are mixed with other substrates, their general effect on overall fermentation can be reduced (Narvaez et al. 2011). *Rheum nobile* and *Carduus pycnocephalus* have been reported to decrease CH_4 production consistently without any apparent adverse effect on other parameters of the rumen fermentation, when they were mixed with other substrate at 1:10 ratio *in vitro* (Bodas et al. 2008). These findings suggest that these bioactive plants can reduce ruminant CH_4 when added with other substrates.

Besides CH_4 mitigation, another potential implication of bioactive plants is that they could also be a valuable component in animal's diet. In Australia, livestock production relies heavily on conventional feed. There is a need to explore local and novel feed resources for animal production with the concern of food security (Place et al. 2009; Alonso-Diaz et al. 2010; Place et al. 2013). However, bioactive plants are not common in animal diets. Their phytochemicals may affect forage intake and animal productivity. Before feeding animals bioactive plants, *in vitro* assessment is required to prevent potential harmful effects to animals. Often, these bioactive plants can not be the sole source of feed for animals because their phytochemicals can be toxic to the host and their total biomass production is often not sufficient to sustain production. It is more practical to incorporate them as a component of the animal's diet to contribute to the feed resource, as well as helping to reduce CH_4 production. Practically, it is essential to achieve low methanogenesis without lowering productivity and profitability and the health of the to animals (Demeyer & De Graeve 1991). To achieve this, *in vitro* studies can be used to select the right proportions of bioactive plants in the diet which can then be confirmed in the *in vivo*. This process is discussed in the next section.

Table 2.2 Examples of effects of different plant extracts on *in vitro* methanogenesis and other in vitro fermentation parameters.

Species	Donors of rumen inoculum; substrate	Effect on CH_4 or methanogens	Other effects on rumen fermentation and cellulolytic bacteria
[1]Tea saponin	sheep; alfalfa hay:concentrate ration (60:40)	Decreased: CH_4 production (8%)	Increased: propionate (11%), *F. succinogenes* (41%); No effects on total VFA production, *R. flavefaciens*, methanogens
[2]*Knautia arvensis* (methanol extract)	Holstein cattle; concentrate: hay (1:1)	Decreased: CH_4 production (5.5-6.4%)	Increased: *Fibrobacter* (24%), *Ruminococcus* (18%); No effects on total VFA, propionate, methanogen
[3]*Quillaja saponaria* (saponins extract)	Holstein cattle; concentrate: hay (1:1)	No effect: CH_4 production	Decreased: ammonia production (21%); Increased: propionate (15-19%); No effect on total VFA
[3]*Trigenella foenumgraecum* seed, (methanol extract)	Holstein cattle; concentrate: hay	Decreased: methanogens (22%), CH_4 production (1.8-2.0%)	Increased: Fibrobacter (42%), ruminococcus (40%) No effect on total VFA
[4]*Yucca schidigera* whole plant powder	Holstein cattle, barley silage : concentrate (51:49)	Decreased: CH_4 production (8-26%)	Decreased: total gas (2-4%), ammonia (81-100%); Increased: propionate (10-36%)

[1]Guo et al. (2008), [2]Goel et al. (2008), [3]Pen et al. (2006), [4]Holtshausen et al. (2009).

2.3.2.2 CH_4 mitigation with plant extracts

Plants produce a diverse array of plant secondary metabolites to protect themselves against herbivore, microbial and insect attack as well as environmental stresses (Wallace 2004). Recently, there have been a number of *in vitro* studies investigating effects of plant extracts and their pathways on ruminal microbial fermentation and CH_4 mitigation (Cardozo et al. 2004; Abdalla et al. 2009; Patra & Yu 2012; Hassanat & Benchaar 2013). Some plant extracts have been reported to have selective or general activity towards the ruminal microbial population (Wallace 2004; Patra & Saxena 2009). The specific activities of most interest were those that altered rumen fermentation and nitrogen metabolism, and CH_4 production and/or nutritional stresses such as bloat or acidosis, which could all improve the productivity and health of animals (Wallace et al. 2002; Kamra et al. 2006; Rochfort et al. 2008; Hutton et al. 2009; Durmic et al. 2012).

The main phytochemicals responsible for low methanogenesis are tannins, saponins and essential oils (Hess et al. 2003; Hart et al. 2008; Kamra et al. 2008; Hook et al. 2010; Patra & Saxena 2010). Saponins are a group of high molecular weight glycosides that have soap-like properties, which are effective antimicrobial agents in a wide array of plants (Osbourn 1996). Condensed tannins are natural, water soluble, plant phenolic compounds with diverse structures that are found abundantly in plants and are capable of binding protein, as well as interacting with microbes in the rumen (McLeod 1974; Robbins et al. 1987; Mangan 1988; McSweeney et al. 2001). Essential oils are the "oil" of the plant, that have been widely evaluated as feed additives for improving microbial metabolism in the rumen (Castillejos et al. 2007; Kamra et al. 2008; Patra & Yu 2012). The effects of these plant extracts on CH_4 mitigation varies according to concentration of extracts, the animal's diet or *in vitro* fermentation substrate and the response of ruminal microorganisms.

The environmental factors and growth stages of plants can affect the concentration of plant secondary compounds produced. For example, *Sesbania sesban* and *Acacia angustissima* were collected at two sites and tested in a continuous *in vitro* fermentation study. Plants grown at lower elevation with less annual precipitation had higher concentrations of saponins and tannins and therefore had lower CH_4 production than plants at the other site (Bekele et al. 2009). The vegetative stage of Purple prairie clover has higher phenolic content in the leaf and inflorescence and therefore had higher *in*

vitro degradability but lower protein degradability than those harvested at flowering stage (Jin et al. 2012). The variability in effects of the same secondary plant compound due to environmental factors and growth stage are difficult to predict, which makes it hard to transfer one finding to another. The diverse nature of plant secondary compounds can also add to the confusion about why, for example, saponins appear to be effective in one case but not in another (Santoso et al. 2004; Holtshausen et al. 2009). Testing well defined, even purified, classes of compounds under controlled environments at different growth stages may be needed to better predict the effects of different secondary compounds from different sources.

Predicting the effects of secondary compounds is complicated by the observation that the effects can also be influenced by the animal diet, *in vitro* incubation substrate and ruminant species being fed. When saponins extracted from *Sesabania sesban* and Fenugreek sp (*Trigonella foenum-graecum* L.) were tested *in vitro* against rumen liquor from Friesian Holstein cattle, they inhibited CH_4 production from concentrate based hay-diets, but not in roughage-based diets (Goel et al. 2008). In a study of commercial *Y. schidigera* extract (Desert King International), adding a supplement of 1.2 g/kg of *Y. schidigera* to an orchard grass silage and concentrate (70:30) diet, decreased CH_4 production per kg DM intake by 10% in sheep after 15 days feeding (Santoso et al. 2004). However, when 10 g/kg of the same supplement was added to a silage-based diet (forage: concentrate = 51:49), CH_4 production was not affected in cattle after 28 days (Holtshausen et al. 2009). The differences in the effects of the same extract may have been due to the different doses used, the species of animal or the base diet fed to the animals. The effect of the *Y. schidigera* extract could be over or underestimated depending on which one of these experiments was used to make the assessment. It is these types of results that highlight the need to be precise about the sources of the extracts, the characterization of the secondary compounds, their purity and dose levels, the fermentation conditions *in vitro*, and composition of the substrate/forage type in the *in vitro* and *in vivo* studies to clarify the cause and effect of different plant secondary compounds in the rumen.

It is difficult to provide a conclusive description on the mechanisms of action of different plant secondary compounds and extracts on CH_4 production, because there is a lack of comprehensive data on the changes in microbial ecology that occur in response to the different secondary compounds separately or in combination. However, plant

extracts appear to reduce CH_4 production via direct inhibition of methanogens, and/or indirectly by reducing number of protozoa and bacteria that affect hydrogen availability for CH_4 formation (Hess et al. 2003; Holtshausen et al. 2009; Patra & Saxena 2010; Feng et al. 2012; Goel & Makkar 2012; Wang et al. 2012). For example, *in vitro,* supplementing tea saponins (53 g/kg) in corn/grass meal (1:1, w/w) reduced CH_4 production by 8%, and increased the population of *F. succinogenes* by 41%, but had no effect on the population of methanogens or *R. flavefaciens* (Guo et al. 2008). In contrast, yucca saponins (1.6 g/kg) incubated with an alfalfa hay and dairy concentrate mixture increased the *R. flavefaciens* population, but had no effect on the *R. albus* population (Patra et al. 2012). Sheep fed a barley-based diet supplemented with chestnut tannins at 30 g/kg for 60 days, had lower CH_4 production (24%) and a lower population of methanogens and protozoa, but there were no changes to the number of *R. flavefaciens*, *R. albus* or *F. succinogenes* (Liu et al. 2011). These are just some examples of studies that highlight variable nature of the effects of plant extracts on modifying ruminal fermentation and microbial ecology. There is a need to try and standardize, or better characterize, the conditions under which different secondary compounds and extracts affect fermentation and CH_4 production. Purifying fractions that contain the targeted activity will improve our understanding of the mechanisms of action of the compounds involved, which may enable better predictions of their effects to be made, as well as narrow down the scope of the search for plants that have the potential to mitigate CH_4.

2.4 Evaluation process from *in vitro* to *in vivo* is essential

Before introducing a new plant to livestock as a feed to reduce CH_4, certain practical considerations such as adaptation of the ruminal microbes, toxicity to ruminants and feed digestibility, need to be assessed. It is thought that bioactive plants can reduce CH_4 production via the direct inhibition of methanogens and/or indirectly by inhibiting other ruminal microbes. Depending on how general the antimicrobial effects towards the other ruminal bacteria and the extent of their inhibition, it is possible that overall inhibition of fermentation may occur and that both CH_4 production and productivity are reduced. Therefore, it is vital to understand the mechanisms of action behind the reduction in methanogenesis to avoid causing an associated reduction in animal production.

An effective process for assessing the potential use and safety of feeding novel bioactive plants with antimethanogenic effects prior to feeding to animals could be to use short-term (batch culture) and long-term (continuous) *in vitro* fermentation to examine the specificity of the antimicrobial effects in the *in vitro*. For example, 24 h batch culture fermentation could be used to confirm the antimethanogenic effects compared to the overall effect on fermentation and how the doses of the novel plants or extracts influences that comparison. The selected plants could then be tested at different doses in a continuous *in vitro* fermentation system (e.g. Rusitec) to examine the persistency of the effect on CH_4 and general fermentation over a longer term (usually 2-4 weeks). The effect on the degradation of dry matter can also be measured *in vitro* and could be used, along with other measurements of overall fermentation (production of total gas and VFA), to gauge whether feeding the plant may have an adverse effect on the animal by disrupting normal fermentation processes. The information from these two *in vitro* systems could then be used to design an *in vivo* experiment with a 'safe' and effective dose of the novel plant to confirm antimethanogenic effect, and to determine feeding values and impacts on animal's health and productivity (Flachowsky & Lebzien 2012). If *in vivo* the experiment confirms that the plant can reduce CH_4 without affecting the animal's health or productivity, then appropriate field-based systems can be developed to incorporate the novel plant(s) into a grazing system.

The batch culture fermentation is a very useful approach to screen a large number of plant samples. It has been used widely to assess the digestibility of feed for over 30 years, as well as examine the effects of feed additives on rumen fermentation, rumen microbes, and to evaluate the extent to which CH_4 production is altered (Menke et al. 1979; Getachew et al. 2005a; Pelve et al. 2012). Batch culture fermentation enables a short-term test (usually 24 h) for a range of plants or plant extracts and is used commonly to derive dose-response curves for investigating a range of suitable levels of substrate substitution that can reduce CH_4 output while maintaining overall rumen fermentation (Busquet et al. 2005; Cardozo et al. 2005; Bodas et al. 2008; Garc á-Gonz ález et al. 2008b; Soliva et al. 2008; Goiri et al. 2009). However, 24 h batch culture fermentation cannot depict the adaptation of ruminal microbes to an introduced feed, which needs days to weeks. It is also a closed fermentation system with no inflow, outflow and absorption, so the products of fermentation accumulate instead of being cleared, which has a feedback effect on the process of fermentation. Therefore, results from 24 h batch culture fermentation should only be considered a first quick and

relatively cheap screening step as a guide to what may happen in an animal, particularly the bioactivity of a novel plant, rather than representing closely what happens in animals (Cardozo et al. 2004).

The limitations of the batch culture fermentation can be minimized by using the Rusitec system. The Rusitec is a continuous culture fermentation that is 'fed' continuously and has both inflow of artificial saliva for buffering and an outflow of fermentation end products, which simulates ruminal fermentation more closely. The Rusitec system provides a stable fermentation for at least two weeks allowing substrates to be tested over a longer term. This means that the persistency of effects and the adaptation of the ruminal microbial community to target plants and substrates can be investigated. The adaptation of ruminal microbes to the introduced plant can be monitored by examining changes in microbial profiles and fermentation products (e.g. total gas, VFA and CH_4). The degradation of dry matter during the fermentation can also be measured to estimate the potential feeding value of the plant to animals. However, the total VFA production and average neutral detergent fibre (NDF) digestibility in Rusitec has been shown to be lower than *in vivo* (Hristov et al. 2012) and the relationship between CH_4 measured *in vitro* and *in vivo* is also weak (R^2 = 0.26; Moss and Givens (1997)). Clearly, there are limitations to the Rusitec, for example the fermentation vessel has no absorptive function, and the conclusions about persistency and adaptation of ruminal microorganisms need to be expressed within the limitations. However, the Rusitec does provide a good bridging step between *in vitro* batch culture fermentation and *in vivo* because it can confirm findings from batch culture fermentation (Czerkawski & Breckenridge 1977) and help design the *in vivo* experiment. It is essential to test any new or novel plant (or product) *in vivo* before making any conclusions about its practical use or implementing anything in the field.

Ultimately, animals need to be fed the bioactive plant or plant product to confirm any antimethanogenic or antimicrobial effects that may have been detected *in vitro*. Animals should be fed the plant or plant product for at least four weeks to allow the microbial population to adapt and then measured in respiration chambers or by sulphur hexafluoride tracer technique (Blümmel et al. 2005; Getachew et al. 2005b; Bhatta et al. 2007; Bhatta et al. 2008). There is no hard and fast rule about the time required for ruminal microbes to adapt to a feed type and there are many different views about how long it should be. However, it has been reported that ruminal microbes can build up

tolerance to the tested supplementation after 20 days (Zhang et al. 2011). For example, ginger powder decreased total VFA for 20 days and then this effect was no longer detected, whereas studies with blended essential oils showed that nitrogen metabolism needed a 4-week adaptation time (Molero et al. 2004; Castillejos et al. 2007). There are numerous other adaptation times reported for different substrates but there is general consensus that four weeks feeding time is desirable to minimise the chance of not detecting the possible adaptation of ruminal microbes to the new substrate.

2.5 *Eremophila glabra* and its potential to reduce CH_4 emission

After screening over 100 Australian native bioactive plant species within the 'Enrich' project, *Eremophila glabra* Juss. (Scrophulariaceae) has received attention because of its antimethanogenic bioactivity (Durmic et al. 2010a). In batch culture fermentation, *E. glabra* produced a significantly higher amount of propionate with a low A:P and less CH_4. This suggests that *E. glabra* promoted hydrogen transfer towards propionate production instead of CH_4 (Durmic et al. 2010a). In addition to its low methanogenic potential, *E. glabra* has over 60% organic matter digestibility *in vitro* (Revell et al. 2013). These benefits lead to the interest in incorporating *E. glabra* into shrub-based grazing systems. However, *E. glabra* also has more general antimicrobial effects on fermentation when it is the sole substrate (Durmic et al. 2010a), which has the potential to reduce animal production. Before introducing *E. glabra* to a grazing system, it is also important to understand the persistency of the antimethanogenic effect, to explore whether *E. glabra* could be fed at a low level and reduce CH_4 without reducing productivity.

Eremophila species (Myoporaceae) grow only in Australia (Chinnock 2007) and are one of the most widely distributed and diverse genera within Australia (Richmond 1993). *Eremophila* species grow mainly in the semi-arid and arid zones of Australia. These shrubs are characterised as drought, fire, frost and grazing tolerant (Frazier 1965; Richmond 1993). There has been an increasing interest in exploring Australian native shrubs as a perennial feed source to develop profitable and more diverse grazing systems for livestock in South Australia and Western Australia, where there is a Mediterranean climate and a feed gap during summer and autumn. The limitation of using shrubs is that their plant secondary compounds have the potential to affect animal productivity (Blache et al. 2008; Durmic & Blache 2012). In the case of *E. glabra*, it could be a valuable component of a forage shrub mix aimed at filling the autumn feed

gap if it can be demonstrated to reduce CH_4 production without adversely affecting animal productivity.

Most *Eremophila* species produce abundant quantities of resin (up to 20% DM): a mixture of lipids, flavones and terpenes in leaves and terminal branches (Ghisalberti 1994a). They are rich in secondary compounds such as essential oils, furanoid sesquiterpenes, and serrulatane diterpenes, which are known for their antimicrobial properties (Ghisalberti 1994b). A number of diterpenes from the leaves and terminal branches of *Eremophila* have been isolated (Forster et al. 1986). Among these diterpenes, the serrulatane diterpenes, which are phenolic compounds, have antimicrobial effects (Ghisalberti 1994a) and may affect rumen fermentation. In addition, extracts of *E. glabra* have exhibited anthelmintic activity against sheep intestinal worms (Kotze et al. 2009) and inhibitory effects on the growth of *Streptococcus bovis*, a gram-positive amylolytic rumen bacterium (Hutton et al. 2012). It is likely that extracts of *E.glabra* can also reduce CH_4 emission.

2.6 General hypothesis and outline of this thesis

In summary, ruminal methanogenesis can be modulated by several factors, including changing an animal's diet. The diet can have either a direct or indirect inhibition on methanogenesis depending on whether there is specific inhibition of the methanogens or on ruminal bacterial species that determine hydrogen availability. Currently, there is an interest in exploring the potential of using Australian native perennial shrubs to develop more diverse, novel grazing systems that can help fill the feed gap that is associated with the Mediterranean climate regions of Australia. *E. glabra* has the potential to be a valuable component of the novel shrub-based systems because it has antimethanogenic properties. However, it has also been established that it has more general antimicrobial properties and can inhibit overall fermentation when it is used as the sole substrate *in vitro* (Durmic et al. 2010a). It has not been established whether there is a level of substrate that can achieve the benefits of lowering CH_4 production without negatively affecting fermentation, nor has any level of *E. glabra* been fed to animals under controlled conditions to examine its effects on productivity and CH_4 production. Sheep are known to graze *E. glabra*, so it is possible that sheep consuming a mixture of *E. glabra* and oaten chaff/lupin, a common supplementary diet for sheep in Western Australia, will produce less CH_4 than without *E. glabra* and still be productive. However, this needs to be tested under controlled conditions. Furthermore, the

mechanisms responsible for low CH_4 production, particularly the effects on rumen microbes and the plant extract(s)/compounds responsible for the bioactivity of *E. glabra* have not been identified.

My overarching hypothesis for this thesis was that *E. glabra* could be fed to sheep as part of a mixed diet to reduce CH_4 production while maintaining animal productivity and health. More specific hypotheses were developed and tested in each of four experiments, which are the basis of the four experimental chapters. The first three experiments were undertaken in a series of experiments with the objective of testing the antimethanogenic and more general antimicrobial effects of *E. glabra* in the *in vitro* (batch culture and Rusitec fermentation) and under controlled *in vivo* conditions. The final chapter explores the organic chemistry for purifying the compounds responsible for the bioactivity.

Chapter 3 *Eremophila glabra* can reduce methane production without reducing rumen fermentation in batch culture

Abstract

Eremophila glabra Juss. (Scrophulariaceae) impairs methane production and overall rumen fermentation when fermented as sole substrate in an *in vitro* batch culture. This study aimed to select appropriate level(s) of *E. glabra* that would decrease methane production without causing adverse effects on ruminal fermentation. *E. glabra* was tested at seven substitution levels by replacing 0.075 to 0.375 g/g of control substrate. Control substrate was 0.5 g oaten chaff/lupin mix: 0.784 g/g oaten chaff, 0.196 g/g lupin and 0.0196 g/g minerals. *E. glabra* treatments resulted in a dose-dependent decrease in total gas, methane, butyrate and ammonia, and in the acetate to propionate ratio. By contrast, concentration of total volatile fatty acids, acetate and propionate was not affected. Total gas production was reduced significantly with *E. glabra* inclusion of more than 0.40 g/g, and methane reduction was significant with more than 0.50 g/g. However, the reduction rate of methane exceeded the rate of total gas reduction, indicating methane production was differentially inhibited by *E. glabra.* In conclusion, using *E. glabra* in a mixed substrate at a substitution rate of less than 50% in an oaten chaff/lupin mix could aid methane mitigation by >10% with least effects on rumen fermentation.

Keywords: Batch culture, *Eremophila glabra*, methane production, dose-response

3.1 Introduction

Methane (CH_4) formation in the rumen is affected by the composition of the diet (Van Kessel & Russell 1996), leading to an avenue for mitigation of emissions (Beauchemin et al. 2008; Eckard et al. 2010; Grainger & Beauchemin 2011). In addition, the reducing CH_4 production could promote other microorganisms that use hydrogen (Chaucheyras-Durand et al. 2010), increasing feed utilization and productivity (Trei et al. 1972; Johnson & Johnson 1995). For this purpose, alternative plants have been investigated as potential feed sources (Soliva et al. 2008; Durmic et al. 2010a). Studies with such plants and their bioactive extracts have confirmed that they can change the pattern of rumen fermentation and thus inhibit methanogenesis (Busquet et al. 2005; Patra et al. 2006; Bodas et al. 2008; Garc á-Gonz ález et al. 2008a; Durmic et al. 2010a).

The Australian plant, *Eremophila glabra* Juss. (Scrophulariaceae), is under investigation as a drought-tolerant grazing fodder in Australia, but it is also a potential antimethanogenic plant (Durmic et al. 2010a). When *E. glabra* was fermented as the sole substrate, CH_4 production was reduced by 43.7%, but it also reduced total gas production and concentration of total volatile fatty acids (VFA), two unwanted side-effects (Durmic et al. 2010a). However, when *E. glabra* was mixed with other substrates, for example, with *Medicago sativa* at a 1:1 ratio, methanogenesis was still reduced, but the total gas production and total VFA were maintained, indicating no effect on overall fermentation (Li et al. 2010).

I hypothesized that *E. glabra* would be equally effective at reducing CH_4 when mixed with other substrates such as an oaten chaff/lupin mix, the most common supplementary diet for sheep in Western Australia. We therefore tested when *E. glabra* mixed with oaten chaff/lupin, it would reduce CH_4 production without disrupting rumen fermentation. The relationship between the amount of bioactive plants or their extracts and CH_4 production is generally dose-dependent (García-González et al. 2008b; Macheboeuf et al. 2008; Goiri et al. 2009), so our initial challenge was to compare rates of substitution to determine the optimum level of inclusion.

3.2 Materials and methods

The use of animals and the experimental protocol were approved by the Animal Ethics Committee of the University of Western Australia (RA/3/100/1097) according to the recommendations of the Australian National Health & Medical Research Council.

3.2.1 Experimental design

Seven levels of substitution of *E. glabra* were tested in 24 h *in vitro* batch culture fermentation. Gas and fermentation liquid samples were collected at the end of fermentation at 24 h. Production of CH_4 was measured and total gas and total VFA were measured as indicators of overall rumen fermentation.

3.2.2 Plant material

E. glabra (accession number SA 45599) were harvested from multiple plants grown at an experimental site under the control of the South Australian Research and Development Institute (SARDI, Waite Institute, South Australia) at reproductive stage

(< 1year old) in February 2008. Leaves were collected, freeze dried, sealed in an evacuated bag and stored at room temperature. Samples were ground (Glen Creston, Stanmore, England) to pass through a 1.0 mm screen before experimentation. Oaten chaff and lupin were sourced from Feedman Stock Feeders (Gosnells, WA, Australia) and minerals were provided by John Milton (Independent Lab Services, WA). Each material was weighed separately, homogenized and ground through a 1.0 mm sieve. The chemical compositions of plant material are shown in Table 3.1.

Table 3.1 Chemical compositions of plant material used in batch culture[a].

	Oaten chaff	Lupin	*E. glabra*
Dry matter (DM, g/kg)	918	910	947
Neutral detergent fibre (NDF, g/kg DM)	565	235	287
Acid detergent fibre (ADF, g/kg DM)	311	197	182
Crude protein (g/kg DM)	57	320	112

[a]Australian fodder industries association (AFIA) standard lab fodder analysis

3.2.3 Treatments

Control substrate was a mixture of oaten chaff (0.784 g/g dry matter (DM)), lupin (0.196 g/g DM) and mineral (0.0196 g/g DM). Seven substitution levels of *E. glabra* were: EG15 (0.15 g/g DM), EG20 (0.20 g/g DM), EG25 (0.25 g/g DM), EG30 (0.30 g/g DM), EG40 (0.40 g/g DM), EG50 (0.50 g/g DM), EG75 (0.75 g/g DM) and the treatments were prepared by substituting the control substrate with the amount of *E. glabra* required for each treatment level. Therefore, the actual amounts of the substitution of *E. glabra* were 0.075, 0.10, 0.15, 0.20, 0.25, and 0.375 g respectively. *E.glabra* was also incubated as sole substrate in this study (EG100, 1.00 g/g DM). Each treatment had three replicates.

3.2.4 Rumen inoculum

Rumen fluid from three fistulated Merino sheep was collected 2 h after morning feeding. The sheep had been fed on an oaten chaff/lupin diet *ad libitum* for two weeks before sample collection with unlimited access to water. Rumen fluid was collected using a vacuum pump, pooled and strained through cheesecloth before being used as the inoculum. After collection, rumen inoculum was transferred to an anaerobic chamber (Coy Vinyl Anaerobic Chamber; Coy Laboratory Products Inc., USA, supplied with 800 mL/L N_2, 100 mL/L CO_2, and 100 mL/L H_2) and buffered to pH 7.2 (McDougall 1948).

3.2.5 In vitro batch culture fermentation

The *in vitro* batch culture was used as modified by Durmic et al. (2010a). Briefly, one day before the experiment, 0.5 g DM of treatment was weighed into 100 mL serum bottles and transferred to 39 ℃ anaerobic chamber to expel the oxygen from the bottles. On the day of the experiment, 50 mL buffered rumen inoculum was dispensed into prepared serum bottles. Each serum bottle was sealed with a butyl rubber stopper and crimped with an aluminum seal and incubated at 39 ℃ with constant shaking at 50 rpm. Each treatment and the controls were prepared in triplicate.

After 24 h incubation, serum bottles were placed in a water bath at 39 ℃, and gas pressure was measured using a pressure transducer (Greisinger Electronic GmbH, Regenstauf, Germany). Total gas production was calculated by transferring gas pressure to standard atmospheric pressure. After measuring gas pressure, gas sample (2 mL) was

pumped into a gas chromatographer (GC; Micro Gas Chromatograph, CP-4900, Varian) for immediate analysis of CH_4 concentration. CH_4 production was then calculated as the CH_4 concentration multipling total gas production. The GC had a 60 m HP-1 capillary column using Helium as the carrier gas. The injector temperature was 50 ℃, and the column was held constantly at 60 ℃ during analysis. Two separate samples (1 mL) of fermentation liquid were collected mixed with 200 μL 1 M NaOH and 200 μL 2 M HCl, and stored at –20 ℃ for analysis of VFA and ammonia concentration. The VFA concentration in the fermentation liquid were quantified using an Agilent 6890 Series GC (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with HP6890 injector, capillary column HP-FFAP (30 m × 0.53 mm × 1.0 μm), flame ionization detector and Hewlett-Packard Chemstation software. Hydrogen was used as a carrier gas at 6.6 ml/min, injector temperature was set at 260 ℃, oven temperature at 240 ℃, and the detector temperature at 265 ℃. The concentration was calculated using an internal standard (3-Methyl Valeric acid) calibration method (GC Separation of VFA C2 - C5 Supelco Bulletin No. 749D). The ammonia concentration in the fermentation liquid was measured using the Berthelot reaction (Zadorojny et al. 1973).

3.2.6 Statistical analysis

To determine the effects of *E. glabra* substitutions on production of CH_4, total gas, and concentration of VFA and ammonia, data were subjected to separate one-way ANOVAs. If ANOVAs were significant, means were separated using Fisher's least significant differences (LSD, $\alpha < 0.05$). Linear or quadratic regression analysis was performed on all fermentation parameters against substitution rates of *E. glabra* to determine the dose-response effects. Significance of fixed regression was declared at $P < 0.05$. Linear and quadratic dose-effects were discussed at $R^2 > 0.5$. All statistic analyses were performed in GenStat (13th Edition, VSN International, 2010).

3.3 Results

3.3.1 Total gas and CH_4 production

Total gas production was significantly reduced at EG40 ($P < 0.05$) and the lowest value was for EG100 (Table 3.2). CH_4 production was highest with the control, EG15, EG20, and EG25 treatments, amongst which there were no significant differences, but CH_4 production was decreased by all other *E. glabra* treatments compared to control value. The antimethanogenic effects were significant at EG50, about 16% lower than control

($P < 0.05$) and at all higher rates of substitution. Thus, total CH_4 production was lowest with EG100 ($P < 0.05$). As the levels of *E.glabra* increased, total gas production fell in a linear fashion ($P < 0.001$), whereas CH_4 production decreased linearly ($P < 0.001$) and quadratically ($P < 0.001$).

3.3.2 VFA and ammonia concentration

E. glabra treatments did not significantly affect total VFA and acetate, propionate concentration (Table 3.2). Butyrate and ammonia concentration were reduced at EG50 ($P < 0.05$). Acetate to propionate ratio (A:P) decreased at EG75 ($P < 0.05$). The lowest values for total and individual VFA and ammonia concentration, and for A:P, were recorded with EG100 ($P < 0.05$). Overall, as the levels of *E. glabra* increased, there were linear and quadratic reductions of A:P and butyrate concentration ($P < 0.001$), a quadratic decrease in propionate concentration ($P < 0.001$) and a linear reduction in ammonia concentration ($P < 0.001$). However, total VFA and acetate concentration showed no linear or quadratic relationship to *E. glabra* supplementation.

Table 3.2 Effects of *E. glabra* substitutions on total gas, CH_4, VFA and ammonia production *in vitro* batch fermentation.

Treatment	Total gas production (mL/g DMi)	CH_4 concentration (mL/ 100mL)	CH_4 production (mL/g DMi)	Acetate (mmol/L)	Propionate (mmol/L)	Butyrate (mmol/L)	Total VFA (mmol/L)	Acetate to Propionate	Ammonia (mg/L)
Control	215^{a}	11.9^{ab}	25.5^{a}	72.2^{a}	27.3	11.5^{a}	112^{a}	2.64^{a}	266^{a}
EG15	213^{a}	11.9^{ab}	25.4^{a}	71.7^{a}	27.1	11.3^{ab}	111^{ab}	2.65^{a}	260^{a}
EG20	213^{a}	12.0^{ab}	25.5^{a}	72.2^{a}	27.0	11.3^{ab}	112^{ab}	2.67^{a}	246^{a}
EG25	209^{ab}	11.0^{ab}	23.1^{ab}	69.7^{ab}	26.3	11.1^{ab}	108^{ab}	2.65^{a}	238^{ab}
EG30	211^{ab}	11.6^{ab}	24.5^{ab}	72.0^{a}	27.1	11.5^{a}	112^{ab}	2.65^{a}	254^{a}
EG40	204^{b}	11.0^{ab}	22.4^{ab}	71.0^{ab}	26.7	11.1^{ab}	110^{ab}	2.65^{a}	233^{ab}
EG50	202^{b}	10.6^{bc}	21.4^{b}	69.3^{ab}	26.4	10.6^{b}	107^{ab}	2.62^{a}	205^{b}
EG75	192^{c}	9.3^{c}	17.8^{c}	68.9^{ab}	28.5	9.0^{c}	107^{ab}	2.42^{b}	145^{c}
EG100	184^{c}	7.8^{d}	14.3^{d}	65.3^{b}	28.6	8.0^{d}	103^{b}	2.29^{c}	138^{c}
SEM	1.83	0.82	0.70	1.22	0.53	0.16	1.80	0.01	6.68
P value	<0.001	<0.001	<0.001	0.015	0.065	<0.001	0.041	<0.001	<0.001
Regression, R^2 (P value)									
Linear	0.94 (< 0.001)	0.86 (< 0.001)	0.88 (< 0.001)	NS	NS	0.85 (<0.001)	NS	0.78 (<0.001)	0.89 (<0.001)
Quadratic	NS	0.90 (< 0.001)	0.91 (< 0.001)	NS	NS	0.93 (<0.001)	NS	0.94 (<0.001)	NS

DMi = dry matter incubated. SEM = standard error of means.

[a-d] Values in the same column with different superscripts are significantly different at $P < 0.05$. Values within parenthesis correspond to P-value for regression. NS: no significant linear or quadratic regressions ($P > 0.05$).

3.4 Discussion

It is clear that mixing *E. glabra* with an oaten chaff/lupin substrate differentially reduced CH_4 production in comparison with its effect on overall fermentation, and the level of *E. glabra* needs to be managed carefully. The reduction in CH_4 production is consistent with our previous study with *M. sativa* (Li et al. 2010). However, I did not find a level of *E. glabra* that reduced CH_4 production specifically – this effect was always accompanied by a reduction in total gas production, signifying an inhibition of fermentation. Importantly, the rate of reduction of CH_4 exceeded the rate of total gas reduction. In addition, total VFA concentration was maintained, with small beneficial changes in the proportions of VFA (more propionate produced). The main adverse effects of including *E. glabra* in the substrate occurred at levels above 50% (EG50) and were minimal below 40% (EG40). These results suggest that *E. glabra* could be included as a fermentation substrate at a level that reduces CH_4 production and has minimal effects on overall fermentation.

Overall, these observations indicate that, at the right level, *E. glabra* causes a slight, non-specific depression in fermentation activity and a shift in the fermentation pattern because of impaired methanogenesis. Furthermore, the reduction in CH_4 output was dose-responsive (linear and quadratic), with progressively greater effects seen as the levels of *E. glabra* increased, up to a maximum of 30% reduction in CH_4 output (EG75). The rate of decrease in CH_4 production was greater than the rate of loss in VFA concentration (the reduction of acetate and increase of propionate), suggesting that the inhibition of CH_4 production was stronger and more specific. These changes imply that a compromise between the detrimental and beneficial effects of *E. glabra* can be established by choosing a suitable level of substitution and that there is likely to be some specific activity towards methanogens as well as broader antimicrobial effects.

In vitro fermentation was modulated by *E. glabra*, resulting in inhibition of total gas at high levels (> EG40). There was a clear linear dose-response on total gas production, similar to the *in vitro* study of *Rheum officinale* and *Frangula albus* (García-González et al. 2008b) for other bioactive plants. Total and individual VFA concentration can also be indicators of the effects on fermentation of a new substrate or additive, particularly in relation to fibre digestion (Wallace et al. 2002; Beauchemin et al. 2008; Martin et al. 2010; Bodas et al. 2012). Similar to the effects of chitosan, total VFA concentration in our study was not reduced by *E. glabra* at the levels tested (Goiri et al. 2009). By

contrast, a diet containing ginger root (*Zingiber officinale*) reduces total VFA concentration, but maintains A:P, pH and ammonia-N concentration (Zhang et al. 2011). Total gas production became significantly lower as the level of *E. glabra* increased but the consistency in total VFA concentration suggests that fibre digestion in the presence of *E. glabra* was maintained. It is possible that the fall in total gas production may have been related to the inhibition of protein degradation, as evidenced by the reduction in ammonia production. Similar results have been reported with tannin rich plants in sheep (Bhatta et al. 2007).

The proportions of specific VFA formed in the rumen is an important predictor of CH_4 production because hydrogen is the major substrate for CH_4 formation (Wolin 1960). Propionate is a hydrogen sink whereas acetate and butyrate are hydrogen sources (Wolin & Miller 1982). The results from the present study suggest that *E. glabra* did not result in accumulation of hydrogen, but caused its diversion to propionate. Propionate concentration was promoted at the expense of acetate and butyrate, suggesting that the presence of *E. glabra* in the substrate increased fermentation efficiency. The changes in the productions of acetate and propionate, leading to the lower A:P compared to control, supports the view that less hydrogen is available for CH_4 production (Moss et al. 2000; Janssen 2010). Thus, the reduction in CH_4 production in response to *E. glabra* appears to be due to the shift in microbial fermentation towards an increase in propionate concentration and the decrease in the A:P. These types of changes in the concentration of the individual VFA have also been reported for chitosan, monensin, garlic and capsicum extracts, all of which shifted VFA profile towards a more efficient route (Richardson et al. 1976; Cardozo et al. 2004; Goiri et al. 2009; Ellis et al. 2012a). The promotion of the alternative hydrogen sink (propionate production) by *E. glabra* is beneficial in terms of CH_4 mitigation (Calsamiglia et al. 2007) and would be expected to enhance productivity of ruminant livestock, or at least maintain it – but this can be tested only *in vivo*.

In vitro studies are useful for examining the effect of plant products on ruminant CH_4 production (Patra et al. 2006; Bodas et al. 2008; Soliva et al. 2008; Durmic et al. 2010a), but extrapolation to *in vivo* situations is rather difficult because of the poor relationship between CH_4 produced *in vivo* and *in vitro* ($R^2 = 0.26$, Moss and Givens (1997)). We need to consider the two main limitations of *in vitro* batch culture. Firstly, batch culture studies may over-estimate the modulatory effect of substrates on rumen fermentation,

mainly because ruminal microbes may build up a tolerance to fermentation substrates after long term fermentation (Cardozo et al. 2004). The effects of some plant extracts on rumen microbial fermentation can disappeare after several days of *in vitro* incubation (Cardozo et al. 2004). Secondly, batch culture accumulates end products because it is a closed system, with zero dilution rate and no outflow of the outputs. Therefore, to test the persistency of effects *in vitro*, the Rusitec (continuous *in vitro* simulation system) is often used, because experiments can be run over weeks rather than days and the system is more representative of a dynamic rumen. The longer term *in vitro* fermentation in the Rusitec can give contrasting results to batch fermentation. For example, bromochloromethane reduced total gas production in the continuous system after two days fermentation, but there was no such change in the batch culture fermentation (Goel et al. 2009). In spite of these limitations, batch culture is useful for examining the processes that modify CH_4 emissions and can reflect *in vivo* conditions. For example, chitosan was observed to have the same effects on fermentation *in vitro* and *in vivo*, although it affected rumen bacteria in different ways (Goiri et al. 2010). To better evaluate *E. glabra* before moving to *in vivo* studies, we need to elucidate the ability of rumen bacteria to adapt to this antimethanogenic additive because the effects of bioactive compounds can be transient (Wallace et al. 2002). The next step is to study *E. glabra* in continuous *in vitro* fermentation.

3.5 Conclusion

Replacing part of the normal diet with bioactive plants may be a cost-effective strategy for mitigating CH_4 because such plants will provide animals with an alternative feed source, reduce CH_4 production and, at the same time, maintain or even increase productivity. The present study has shown that *E. glabra* has dose-related antimethanogenic effects when mixed with oaten chaff/lupin, and suggests that *E. glabra* would have a beneficial effect via decreasing A:P ratio, diverting hydrogen to other hydrogen sink and maintaining total VFA concentration (fibre degradation), whilst reducing CH_4 emissions. Understanding the effects of *E. glabra* on rumen fermentation could help us to find alternative feed sources with antimethanogenic effect. The present study has also produced an estimate of the optimum rate of *E. glabra* inclusion for future studies: below EG50.

Chapter 4 *Eremophila glabra* reduces methane production and methanogen populations when fermented in a Rusitec

Abstract

Eremophila glabra Juss. (Scrophulariaceae), a native Australian shrub, has been demonstrated to have low methanogenesis potential in a *in vitro* batch fermentation system suggesting that it may have value as a forage in ruminant livestock systems. The present study used an *in vitro* continuous culture system (Rusitec) to test long-term effects of *E. glabra* on rumen fermentation characteristics, particularly methane production and the methanogen population. The control fermentation substrate was based on oaten chaff and lupin grain. *E. glabra* was substituted at 150, 250, or 400 g/kg DM (EG15, EG25, EG40). Overall, the experiment lasted 33 days, with 12 days of acclimatization, followed by two periods of measurement of fermentation characteristics: total gas, production of methane, concentration of volatile fatty acids, dry matter disappearance and rumen pH. The number of copies of genes specifically associated with total bacteria and cellulolytic bacteria (16S rRNA) and total ruminal methanogenic archaeal organisms (the methyl coenzyme M reductase gene, mcrA) were also measured using quantitative real-time PCR. Production of total gas, methane and volatile fatty acids were significantly reduced by the addition of *E. glabra* in a dose-dependent manner. Treatment EG40 was withdrawn after 7 days because fermentation was almost completely inhibited. At the end of the experiment, the overall methane reduction was 32% for EG15 and 45% for EG25, compared to the control. There were no changes in dry matter digestibility in all the treatments. Total bacterial numbers did not change, but the total methanogen population decreased by up to 42.1% (EG40) when compared to the control substrate. The *Fibrobacter succinogenes* population was reduced at all levels of *E. glabra*, while the *Ruminococcus albus* population was reduced only by EG40. In conclusion, replacing a portion of a fibrous substrate with *E. glabra* causes major reductions in methanogen populations and in methane production over three weeks with some minor inhibition on overall fermentation at the lower inclusion levels. These observations with a continuous *in vitro* culture system add further evidence for the value of *E. glabra* in efforts to mitigate methane emissions form livestock.

Keywords: Rusitec, methane production, methanogen, cellulolytic bacteria

4.1 Introduction

Ruminants are the most prevalent source of methane (CH_4) in Australian agriculture, contributing about 65% of agricultural emissions and approximately 9% of national green house gas emissions (National Greenhouse Gas Inventory 2011). CH_4 is produced during microbial fermentation of feed in the rumen. When cellulolytic bacteria break down carbohydrates to volatile fatty acids (VFA), hydrogen and carbon dioxide (CO_2) are released and then utilized by methanogens to produce CH_4 (Hungate 1966; Demeyer & Van Nevel 1975). The amount of CH_4 produced is regulated by the number of hydrogen-producing and hydrogen-utilising microbial species present in the rumen. Acetate, butyrate, and propionate are major end products from separate metabolic pathways in rumen fermentation. During formation of acetate and butyrate from pyruvate, hydrogen and CO_2 are released, whereas formation of propionate from pyruvate sequestrates hydrogen (Moss et al. 2000). Therefore, propionate concentration and methanogenesis are competitive pathways of hydrogen utilization (Wolin & Miller 1982; Hegarty & Gerdes 1999; Moss et al. 2000). By manipulating the microbial ecology and the balance among these fermentation processes towards greater propionate concentration, it may be possible to alter CH_4 production.

Bioactive plants have been considered 'natural' manipulators of rumen fermentation (Wallace et al. 2002). Results from recent screening projects have suggested that several plants with bioactive secondary compounds can change the activity of rumen microbes and inhibit methanogenesis, for example, *Rheum officinale* and *Frangula alnus* (Garcá-González et al. 2008a; Garcá-González et al. 2008b), *Rheum nobile* (Bodas et al. 2008), and *Terminalia chebula* (Patra et al. 2006). An Australian native shrub, *Eremophila glabra* Juss. (Scrophulariaceae), has the potential to be used as a forage shrub in novel grazing systems (Vercoe et al. 2009), due to its tolerance to drought, fire and grazing (Mitchell et al. 1988). It can also selectively inhibit ruminal bacteria, modulate VFA concentration s and inhibit CH_4 emissions *in vitro* (Hutton et al. 2009; Durmic et al. 2010a; Hutton et al. 2012). However, it almost completely inhibits fermentation when it is used as the sole substrate in an *in vitro* batch fermentation (Durmic et al. 2010a). This negative effect can be moderated without losing the beneficial effects of inhibiting CH_4 production when *E. glabra* is mixed with another substrate, *Medicago sativa*, in 24 h *in vitro* batch culture (Li et al. 2010). These results show that *E. glabra* has the potential to

reduce CH_4 emissions from ruminants if it is consumed as a component of the diet, rather than the sole component.

It is essential to screen potential antimethanogenic plants *in vitro* before testing them *in vivo* because animal experimentation is expensive and there is a duty of care to test the safety of new plants proposed for animal consumption (Revell & Revell 2007). As an interim step between batch culture and animal experimentation, the continuous *in vitro* fermentation system (Rusitec) is the best approach for confirming bioactivity and the persistency of effects on microbial fermentation. In the present study, I used the Rusitec system to examine the persistency of the antimethanogenic effect, and assessed the more general inhibitory effects of *E. glabra*, with the aim of establishing an appropriate and safe amount of *E. glabra* for testing *in vivo*. I hypothesised that *E. glabra* would have a dose-related effect on methanogenesis that would persist *in vitro* during continuous fermentation in a Rusitec.

4.2 Materials and methods

The use of animals and the experimental protocol were approved by the Animal Ethics Committee of the University of Western Australia (RA/3/100/1097) according to the recommendations of the Australian National Health & Medical Research Council.

4.2.1 Experimental design

Continuous *in vitro* fermentation was conducted using a Rusitec. Three levels of *E. glabra* were tested and compared to a control substrate. The fermentation period lasted 33 days, with a 12-day stabilization period on the control substrate, followed by 21 days of incubation with the treatments, beginning on Day 0. Gas samples were collected daily in two measurement periods (Days 1-6 and Days 10-21) to measure CH_4 and total gas, while fermentation liquid was collected daily in period Days 1-6 and every second day during Days 10-21 for VFA, ammonia, and rumen pH. Fermentation liquid samples were collected for molecular microbial analysis one day before and one day after adding *E. glabra*, and then every five days until the completion of the trial (*i.e.* Days -1, 1, 6, 11, 16 and 21). The DNA samples were analysed using quantitative real-time PCR to examine changes in ruminal microbial populations. Treatment EG40 was withdrawn on Day 7 because fermentation was almost completely inhibited: the rumen fluid became dark green, the pH was above 6.8 and very little gas was being produced. The pH of EG15 and EG25 fell on Day 6, so the buffer infusion rate was adjusted from 53mL/h to

56mL/h on Days 7, 8 and 9 until the system was stabilised, and samples from these days were not included in analyses.

4.2.2 Plant material

E. glabra (accession number SA 45599) were harvested from multiple plants grown at an experimental site under the control of the South Australian Research and Development Institute (SARDI, Waite Institute, South Australia) at reproductive stage (< 1year old) in February 2008. Leaves were collected, freeze dried, sealed in an evacuated bag and stored at room temperature. Unground whole dried leaves were used in this study to prevent samples leaking from the nylon bags used.

4.2.3 Preparation of rumen inoculum

Rumen fluid was collected from five fistulated Merino wethers (mean body weight 65.4 ± 2.0 kg) that had been fed *ad libitum* for two weeks on a diet that matched the control substrate for *in vitro* studies (oaten chaff, lupin seed and mineral mix) with unlimited access to water. Rumen fluid was then collected 2 h after morning feeding using a vacuum pump and a wide bore tube that allowed collection of both liquid and solid phase. Rumen samples were kept in pre-warmed thermos flasks, pooled, and strained through 3 layers of cheesecloth to separate the liquid and solid portions before being used as the source of inoculum in the Rusitec.

4.2.4 Treatments

The control substrate contained 784 g/kg dry matter (DM) oaten chaff, 196 g/kg DM lupin grain and 20 g/kg DM minerals. The treatments were prepared by substituting a proportion of control substrate with *E. glabra* at three levels: EG15 (150 g/kg DM), EG25 (250 g/kg DM) and EG40 (400 g/kg DM). Each treatment had three replicates (n = 12 fermentation vessels) and all treatments were assigned to the fermentation vessels randomly. The compositions and energy values for each treatment is presented in Table 4.1.

Table 4.1 Compositions and energy values for each treatment tested in Rusitec.

	Control	EG15	EG25	EG40
Ingredient compositions				
Lupin grain (%)	19.7	16.7	14.7	11.8
Oaten chaff (%)	78.3	66.7	58.8	47.2
E. glabra (%)	0.0	15.0	25.0	40.0
Mineral (%)	2.0	1.7	1.5	1.2
Analytical compositions				
Gross energy (MJ/kg)	18.0	18.1	18.1	18.2
Metabolizable energy (MJ/kg)	9.7	9.8	9.8	9.8
Crude protein (g/kg DM)	118	117	117	116

4.2.5 Rusitec fermentation

Two sets of the same model of Rusitec fermenters manufactured in-house (UWA Combined Workshop, The University of Western Australia, Australia) were used. Each set was equipped with six fermentation vessels (2000 mL overall volume with 1800 mL effective working volume), submerged in a 39 ℃ water bath, and set up for the experiment following the incubation procedure as described by Czerkawski and Breckenridge (1977). On the first day, each vessel contained 500 mL of the strained rumen liquid and 1200 mL of artificial saliva (pH 8.23; McDougall (1948) and four nylon bags. One nylon bag was filled with solid rumen contents (about 23 g wet weight) and the other three bags contained control substrate (15 g DM per day). From Day 2 onwards, the nylon bags containing the solid rumen contents were replaced with a bag containing 15 g DM control substrate. Each vessel was 'fed' daily with 2 bags containing 15 g DM of the substrate at 0930 h and each bag was incubated in the vessel for 48 h. The artificial saliva was prepared daily and infused into the vessels to maintain pH throughout the fermentation period. The dilution rate of artificial saliva was 53-56 mL/h. After every exchange of feedbags, the fermentation vessels were flushed with gaseous N_2 for 1 min to maintain anaerobic conditions. During the stabilization period (12 days), the feed substrate in the nylon bags consisted solely of the control substrate. The stability of the Rusitec system was assessed daily by observing the colour and odour of the fermentation fluid, and by measuring the pH of the fermentation liquid and the gas production before ‘feeding’. The system was considered stable when the gas

production was between 1450 and 1650 mL/d and pH remained consistent within the range of 6.51 and 6.55 in all fermentation vessels. The *E. glabra* treatments were introduced into fermentation vessels over two days (deemed as Day 0 and 1). On Day 0, only half of the full amount of *E. glabra* for each treatment was added, and the full amount was then added in on Day 1.

4.2.6 Sample collection and analysis

4.2.6.1 Total gas and CH_4 productions

All of the gas produced was collected continuously into a gas-sampling bag (wine cask bladder, Scholle Industries) and the bag was replaced daily before feeding. The gas-sampling bag was connected directly to a gas chromatograph (GC; Micro Gas Chromatograph, CP-4900, Varian) for CH_4 analysis. The method was described previously in Chapter 3. The instrument had a 60 m HP-1 capillary column using Helium as the carrier gas. The injector temperature was 50 ℃, and the column was held constantly at 60 ℃ during analysis. Gas sample (2 mL) was pumped into the GC for immediate analysis of CH_4 concentration. CH_4 production was then calculated as the CH_4 concentration multiplied by the total gas production and expressed as total CH_4 produced in mL/g DM.

Following this, the volume of the gas in the bag was measured using a Büchi Vac V-500 water pump (Büchi Labortechnic AG CH-9230 Flawil, Switzerland). The gas was then pumped into a container filled with water, from which water was pushed into a second empty container where the change in volume could be measured as used as an estimate of daily gas production (mL/d). The outflow of fermentation liquid, equal to the buffer infusion rate, was collected continuously into a glass flask containing 20 g NaOH to stop secondary fermentation. This flask was emptied daily and the volume of the outflow liquid was recorded. The total gas production was calculated as daily gas production minus outflow liquid volume and expressed as mL/d.

4.2.6.2 pH, volatile fatty acids and ammonia concentration

In each vessel, pH was measured every morning before feeding using a pH meter (Phtestr20, Eutech instruments). The measurement of VFA and ammonia was described previously in Chapter 3.

4.2.6.3 Disappearance of dry matter

The residue remaining in the nylon bags after 48 h fermentation was collected, washed under running distilled water and dried at 65 ℃ for 48 h, so the residue weight of DM could be recorded. This weight was used for calculation of DM disappearance (DMD) over a period of 48 hours using the formula below:

$$\text{DMD (g/kg)} = (1 - \text{Residue DM/Feed DM}) \times 100.$$

4.2.6.4 DNA extraction and real-time-PCR assay

Samples (10 mL) of fermentation liquid were taken from each vessel 3 h after the addition of substrate, placed into sterile tubes and stored at -20 ℃ for rumen microbial profiling. The DNA was extracted using a modification of the method of Denman and McSweeney (2006). Briefly, genomic DNA was extracted using a bead-beating method, the QIAamp DNA Mini Kit (Qiagen) and the Fast Prep Instrument (Q-BIO gene, Quebec, Canada, Denman & McSweeney 2006). A quantitative real-time PCR assay using specific primers was applied to estimate the numbers and population changes of total bacteria, total methanogens and three major ruminal cellulolytic bacteria (Table 4.2): *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. The real-time PCR assays were performed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, USA), using the methods described by Denman and McSweeney (2006) and Denman et al. (2007). The real-time PCR assays were performed using a Platinum SYBR green quantitative PCR Super Mix-UDG (Invitrogen, USA) and assays were set up using the reaction mixture (35 uL) comprised of a Platinum SYBR green mix (17.5 uL), the primer (forward and reverse, 3.5 uL each), Rox (0.7 uL), distilled water (8.3 uL) and the DNA template (5 uL). The real-time PCR assays were conducted with the following cycle conditions: one cycle at 50 ℃ for 2 min and 95 ℃ for 2 min for initial denaturation, followed by 40 cycles at 95 ℃ for 15 s and 60 ℃ for 1 min for primer annealing and product elongation (Denman et al. 2007). Dissociation curve analysis of PCR end products was performed at 95 ℃ for 2 min and 60 ℃ for 15 s. Total microbial DNA was diluted to 1:20 prior to the use in real-time PCR assays. Amplification efficiencies for the methanogens and bacteria qPCR assay were determined on plasmid DNA described by Denman et al. (2007). Standard curves for the absolute quantification of *F. succinogenes*, *R. flavefaciens* and *R. albus* were derived using the method of Denman and McSweeney (2006). Briefly, standard

curves were obtained using DNA from *F. succinogenes* S85, *R. flavefaciens* Y1and *R. albus* grown overnight in culture at 39 ℃. DNA was then extracted. The copies of these microbes were calculated based on their DNA concentrations and base pair sizes. DNA was diluted to generate eight concentrations over a 4 log dilution range.

Table 4.2. Primer sets used to amplify methyl coenzyme A reductase gene (mcrA) from methanogens and total bacteria, and 16S rRNA sequences from celluloytic bacteria (Denman & McSweeney 2006; Denman et al. 2007; Kang et al. 2010)

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

4.2.7 Statistical analysis

All statistical analyses were performed using GenStat (13th Edition, VSN International, 2010). The data were analysed using one-way ANOVA with treatments as factors to compare the initial variation between four treatments. The data obtained from the treatment period (Days 2-6 and Days 10-21) were analysed using the REML procedure for repeated measures. The model included treatments and day as fixed factors, and fermentation vessel as random effect. The interaction between treatments and day was included. When the interaction between *E. glabra* treatment and day was significant, simple linear regression with groups (i.e. treatments) was used to estimate the differences between treatments within the each period. Least square means and standard error of the means are presented.

To determine if the effects on CH_4 and total gas production, and methanogen populations were dose related, data were subjected to simple linear regression separately. The relationship between methanogen population and CH_4 production was determined by Pearson correlation. For the microbial ecology analysis, microbial numbers were transformed to a $\log_{10}$ scale prior to analysis. Data from different collection points were used as factors in a one-way ANOVA to compare the variations between four treatments. When ANOVAS were significant, means were separated using Fisher's least significant difference (LSD, $P < 0.05$).

4.3 Results

On Day 0, before the application of *E. glabra* treatments, there were no significant differences amongst fermentation vessels for pH or production of total gas and CH_4, and concentration of VFA or ammonia. The major general inhibitory effects of EG40, which led to the discontinuation of this treatment on Day 6, dominated fermentation during Period 1. The removal of EG40 from the experiment provided an opportunity to adjust the buffer infusion rate on Days 7, 8 and 9 to stabilise the system so the assessment of EG15 and EG25 could be continued for longer. For this reason, the results for Period 1 (Day 2-6) are presented briefly here and more focus is given to the longer-term effects of treatments EG15 and EG25 in Period 2 (Day 10-21).

4.3.1 Fermentation in Period 1 (Days 2-6)

Total gas production was reduced, from 1600 mL in the control to 940 mL in EG40, in a linear fashion as the amount of *E. glabra* increased ($R^2 = 0.87$, $P < 0.001$, Table 4.3). The reduction in CH_4 was also evident as a linear relationship with increasing amounts of *E. glabra* ($R^2 = 0.96$; $P < 0.001$).

The pH was higher in *E. glabra* treatments than the control ($P < 0.05$, Table 4.3) and this did not change throughout Period 1. EG15 did not alter total VFA or acetate and propionate concentration, but reduced butyrate concentration ($P < 0.05$). In contrast, EG25 reduced total VFA and acetate and butyrate concentration ($P < 0.05$), but had the highest propionate concentration compared to the control. Total and individual VFA concentration was lowest in EG40 ($P < 0.05$), except for the propionate production, which was higher than the control. DMD was only reduced by EG40, compared to the control ($P < 0.05$). Ammonia concentration was lower in *E. glabra* treatments compared to the control ($P < 0.05$) and could not be detected in EG40 from day 4 until it was discontinued on Day 6.

4.3.2 Fermentation Period 2 (Days 10-21)

4.3.3 Total gas and CH_4 production

The total gas production was significantly lower in the EG treatments compared to the control throughout the period, although it increased gradually over time in the EG25 treatment ($P < 0.05$; Figure 4.1, Table 4.3). Total gas production was affected by *E. glabra* in a dose-related manner ($R^2 = 0.63$; $P < 0.001$). There was also a linear reduction in CH_4 with increasing amounts of *E. glabra* during this period, ($R^2 = 0.82$; .$P < 0.001$). Total gas production was reduced, from 1600 mL in the control to 940 mL in EG40 The reduction in CH_4 production was maintained over time in EG15 but increased over the 12 days in EG25 compared to control values ($P < 0.05$).

4.3.4 pH and VFA concentration

For all *E. glabra* treatments, the pH was higher than control values, and this difference was maintained throughout this period ($P < 0.05$, Table 4.3). Total VFA and individual acetate and butyrate concentration was reduced by *E. glabra* but propionate concentration was increased ($P < 0.05$, Table 3). The individual VFA concentration

varied with time and the degree of reduction in total and individual acetate and butyrate concentration, as well as the increase in propionate concentration, in relation to the substitution of *E. glabra* (Figure 4.1).

4.3.5 Dry matter disappearance and ammonia concentration

There were no differences in DMD among treatments but ammonia concentration was reduced by all *E. glabra* treatments ($P < 0.05$, Table 4.3). The degree of the reduction in ammonia concentration varied over time ($P < 0.05$).

4.3.6 Ruminal microbial populations

4.3.6.1 Total bacterial population

On Day 0, the average total number of bacteria was at 9.81 ± 0.06 $\log_{10}$ cells/mL. There were no significant differences in total bacterial numbers among treatments throughout the incubation period (Table 4.4).

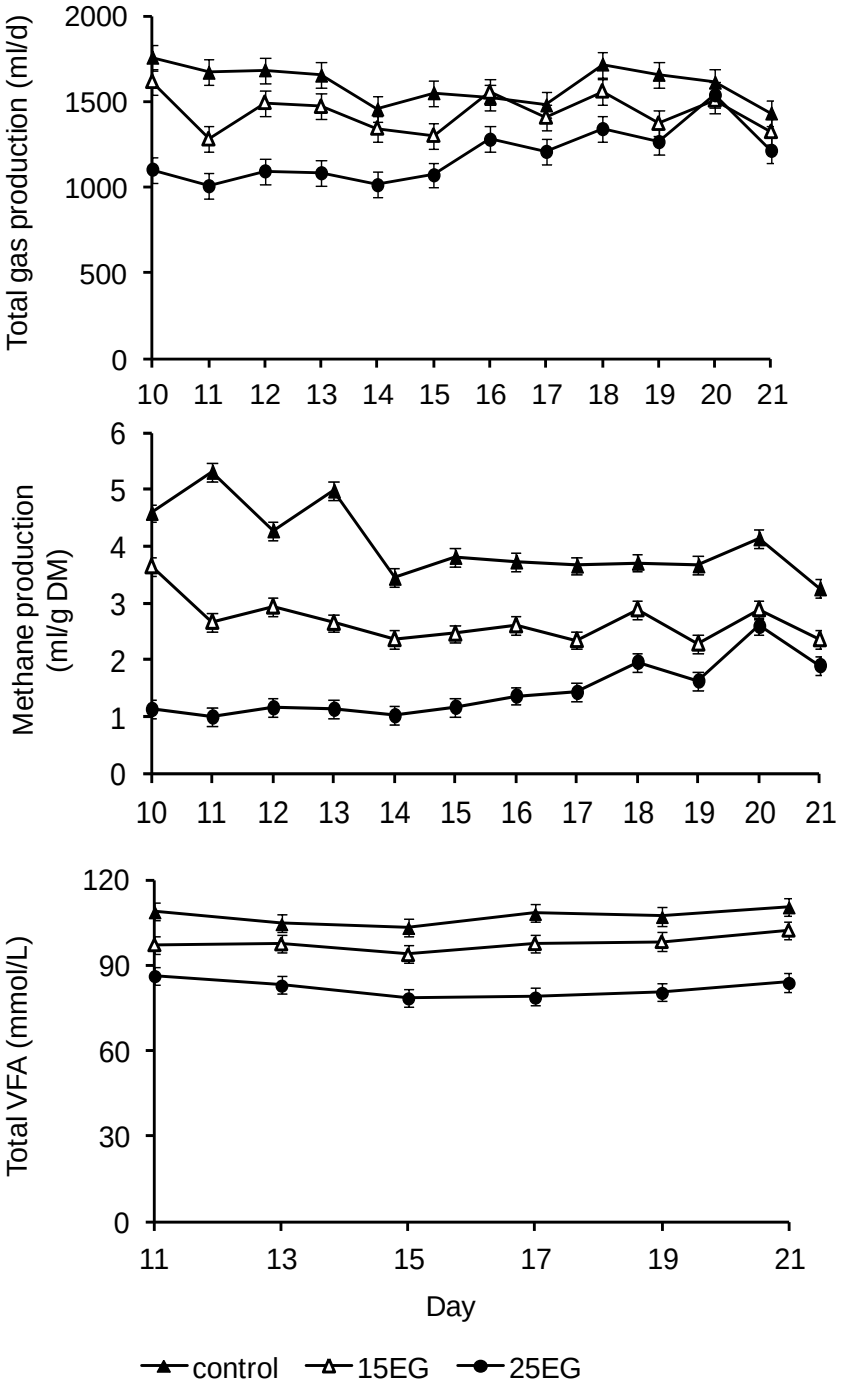

Figure 4.1. Total gas, CH_4 and VFA production in fermentation vessels (Rusitec system) in response to control (▲), EG15 (Δ) and EG25 (●) treatments from Day 10 to 21. The error bars represent the standard error of the means (n = 3).

Table 4.3 Fermentation parameters (mean ±SEM) in control and EG15, EG25 and EG40 treatments in day 2-6 and day 10-21.

	Contr	EG15	EG25	EG40	SEM	P		
			Day 2-6			Treatment	Day	T ×D
pH	6.50[c]	6.58[b]	6.57[b]	6.75[a]	0.02	<0.001	0.006	0.016
Total gas production (mL/d)	1618[a]	1536[b]	1251[c]	943[d]	26.7	<0.001	0.282	0.722
Methane production (mL/g DMi)	5.36[a]	3.52[b]	2.47[c]	1.00[d]	1.7	<0.001	0.151	0.219
Total VFA (mmol/L)	105[a]	98[a]	95[b]	76[c]	2.7	0.001	0.044	<0.001
Acetate (mmol/L)	64.4[a]	59.0[a]	55.7[b]	42.5[c]	1.53	<0.001	0.009	<0.001
Butyrate (mmol/L)	17.4[a]	14.7[b]	11.6[c]	8.6[d]	0.60	<0.001	0.004	0.004
Propionate (mmol/L)	23.3[c]	24.4[c]	28.1[a]	24.6[b]	0.79	0.031	0.049	0.007
Acetate:propionate	2.70[a]	2.44[b]	1.94[c]	1.70[d]	0.04	<0.001	<0.00	0.008
Ammonia (mg/L)	166[a]	97[b]	77[c]	2[d]	7.6	<0.001	0.452	0.951
DMD (%)	70.4[a]	73.2[a]	73.4[a]	59.5[b]	1.37	<0.001	0.002	0.933
			Day 10-21					
pH	6.29[c]	6.41[b]	6.61[a]	-	0.01	<0.001	0.313	0.939
Total gas production (mL/d)	1602[a]	1438[b]	1187[c]	-	42.5	<0.001	0.069	0.024
Methane production (mL/g DMi)	3.69[a]	2.56[b]	1.91[c]	-	1.58	<0.001	<0.001	0.015
Total VFA (mmol/L)	107[a]	98[b]	82[c]	-	1.2	<0.001	0.074	0.818
Acetate (mmol/L)	64.4[a]	55.2[b]	47.0[c]	-	0.53	<0.001	0.003	0.993
Butyrate (mmol/L)	19.1[a]	16.9[b]	6.8[c]	-	0.46	<0.001	0.005	0.258
Propionate (mmol/L)	23.6[c]	26.2[b]	28.2[a]	-	0.55	0.03	0.045	0.023
Acetate:propionate	2.70[a]	2.13[b]	1.70[c]	-	0.04	<0.001	0.003	0.008
Ammonia (mg/L)	161[a]	94[b]	71[c]	-	7.7	<0.001	<0.001	0.129
DMD (%)	73.2[a]	71.7[a]	71.8[a]	-	0.72	0.003	<0.001	0.003

*Treatment EG40 was stopped on day7. SEM = standard error of means.

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

4.3.6.2 Methanogen population

On Day -1, there were similar numbers of methanogens in each vessel (Table 4.4). Following addition of *E.glabra,* and at each sampling time thereafter, all *E. glabra* treatments had lower numbers of methanogens compared to the control ($P < 0.05$). In the first period, the lowest numbers occurred in EG40 and the decrease in methanogen numbers was related to the substitutions of *E. glabra* ($R^2 = 0.61$; $P < 0.001$). Methanogen numbers in the EG treatments gradually increased at each sampling time during the second period but were still lower than the control on Day 21. Although the numbers of methanogens in EG25 were initially lower than in EG15 ($P < 0.05$), they were the same on Day 21 (Table 4.4). Overall, methanogen population numbers were not correlated to CH_4 production ($r = 0.343$).

4.3.6.3 Cellulolytic bacterial populations

On Day -1, there were no differences among treatments in the numbers of cellulolytic bacteria (*F. succinogenes* and *R. albus,*Table 4.4). *F. succinogenes* populations were reduced in all *E. glabra* treatments during the experimental period ($P < 0.05$), whereas *R. albus* populations increased after 24 h incubation with *E. glabra,* ($P < 0.05$), gradually declined on Days 6 and 11, but then increased again over the remainder of the sampling period. On Day 16, *R. albus* populations in EG15 and EG25 treatments were comparable to control values and continued to increase to be higher than control values on Day 21 ($P < 0.05$). *R. flavefaciens* numbers were below the detection threshold of real-time PCR in all samples.

Table 4.4. Number of methanogens, *F. succinogenes*, and *R. albus* in fermentation liquid (log10 cells/mL).

	Day	Control	EG15	EG25	EG40*	SEM
Total bacteria	0	10.2	10.0	9.8	9.8	0.09
	1	9.9	9.9	9.9	9.9	0.01
	6	10.1	9.8	9.9	9.9	0.06
	11	9.9	9.9	10.0	-	0.05
	16	9.9	9.8	9.9	-	0.03
	21	9.9	9.8	9.9	-	0.02
Methanogen	0	8.03	8.01	8.11	8.06	0.03
	1	8.21^{a}	8.05^{b}	7.82^{c}	7.76^{c}	0.04
	6	8.15^{a}	8.12^{a}	7.97^{b}	7.72^{c}	0.02
	11	8.37^{a}	8.19^{b}	7.96^{c}	-	0.02
	16	8.41^{a}	8.27^{b}	8.27^{b}	-	0.02
	21	8.52^{a}	8.25^{b}	8.29^{b}	-	0.03
Fibrobacter succinogenes	0	6.28	6.41	6.30	6.31	0.04
	1	6.32^{a}	5.21^{b}	4.24^{c}	3.82^{d}	0.09
	6	6.35^{a}	5.46^{b}	4.28^{c}	3.31^{d}	0.16
	11	6.02^{a}	4.94^{b}	4.58^{b}	-	0.15
	16	5.85^{a}	5.15^{b}	4.40^{c}	-	0.05
	21	6.07^{a}	5.01^{b}	4.43^{c}	-	0.03
Ruminococcus albus	0	7.22	7.25	7.39	7.24	0.04
	1	7.30^{c}	7.49^{b}	7.56^{a}	7.61^{a}	0.02
	6	7.27^{b}	7.44^{a}	7.11^{c}	6.67^{d}	0.06
	11	7.14^{a}	6.86^{b}	6.88^{b}	-	0.04
	16	7.09	7.25	7.10	-	0.06
	21	7.08^{b}	7.29^{a}	7.43^{a}	-	0.05

[a-d] Values in a same row with different superscript letters are significantly different at $P < 0.05$.
SEM = standard error of means.
*EG40 treatment was stopped on day 7.

4.4 Discussion

E. glabra has a dose-related effect on methanogenesis and the effect persists for 3 weeks during continuous fermentation in a Rusitec system. Based on the changes in the microbial populations, the elevated levels of propionate and the consistently lower A:P, the reduction in CH_4 production appears to be the result of both direct inhibitory effects on the methanogens as well as an indirect effect on other ruminal bacterial species. However, the total gas production and CH_4 production gradually increased over the experimental period in the EG25 treatment, indicating that there was some adaptation of the microbial population to *E. glabra*. This adaptation was reflected in the patterns of change of the methanogen and cellulolytic populations examined. Even though there were fluctuations in the number of methanogens and cellulolytic species, the total bacterial number and dry matter disappearance (DMD) did not change throughout the experiment for the control or EG15 and EG25 treatments. This suggests that it may be possible for animals to consume *E. glabra* as minor component of their diet safely and reduce CH_4 emissions, but maintain a healthy microbial fermentation in the rumen.

Numerous plants have been investigated *in vitro* for CH_4 mitigation (Kurihara et al. 1999; DeRamus et al. 2003; Busquet et al. 2005; Cardozo et al. 2005; Bodas et al. 2008; Garc á-Gonz ález et al. 2008a; Soliva et al. 2008; Durmic et al. 2010a; Kennedy & Charmley 2012), but only a few have been tested in the Rusitec system to confirm whether their effects persist without having more general harmful effects on rumen fermentation (Garc á-Gonz ález et al. 2008b; Garc á-Gonz ález et al. 2010). Our earlier results with the *in vitro* batch fermentation system show that *E. glabra* inhibits both methanogenesis and overall fermentation when used as the sole source of substrate (Durmic et al. 2010a), but the negative inhibitory effect on overall fermentation can be moderated when it is used in combination with other substrates over a 24 h period (Chapter 3; Li et al. (2010)). Our results with the Rusitec confirm these earlier observations. Moreover, as the amount of *E. glabra* included as substrate increased, there was a linear reduction in CH_4 production, but also in total gas production and total VFA production. At the highest level of inclusion (40%), fermentation was completely disrupted to the point where the treatment had to be removed from the experiment. There was significantly less total gas, VFA and CH_4 produced, but DMD was also significantly lower and ammonia concentration had almost completely ceased. I also observed general differences in VFA concentration in the two lower *E. glabra*

treatments, illustrated by the lower total VFA concentration compared to the control, with the exception of EG15 until Day 6. After 3 weeks, both CH_4 production and total VFA concentration in the EG15 treatment were significantly lower than the control, suggesting that there may be some penalty on the concentration of VFA produced even at low levels of *E. glabra*. Despite the reduction in VFA concentration there was no reduction in the disappearance of the substrate, which is an indication of the extent to which the substrate is hydrolysed by the microbes. Clearly, *E. glabra* has general antimicrobial effects and there is a tradeoff between the impact of *E. glabra* on CH_4 production and the general inhibitory effects on overall fermentation. However, this negative effect does not appear to reduce the breakdown of substrate.

A reduction in CH_4 production can be a result of direct inhibition of methanogens or a more general effect on the ruminal microbial community (Moss et al. 2000). The consequences of both of these pathways are a change in microbial numbers and end products of fermentation. The reduction of CH_4 production in this study was associated with a reduction in the methanogen population and differences in the VFA production. The methanogen numbers in the *E. glabra* treatments remained significantly lower than the control for three weeks, but the correlation between CH_4 production and methanogen numbers was not significant. It has been suggested that there is not always a direct correlation between CH_4 production and methanogen numbers (Zhou et al. 2011; Danielsson et al. 2012), and that methanogen populations considered as minor species, may play a significant role in methanogenesis when more dominant species are inhibited (Wright et al. 2004). The aim of this *in vitro* experiment was to establish the dose and persistency of the effects of *E. glabra* on microbial fermentation to assess the need and safety of testing it *in vivo*, not to undertake a detailed molecular microbial profiling study, which can be of limited value from samples taken *in vitro*.

Manipulating the balance among individual VFA is one way CH_4 production can be altered (Wolin 1960). Reducing hydrogen production or diverting hydrogen to non-methanogenic pathways, is reflected by changes in overall fermentation end products, the ratio of propionate to acetate, as well as the changes in the number of other ruminal microbial species (Wallace et al. 1981). In our study, the profiles of individual VFA changed in relation to the amount of *E. glabra* used as substrate, and there was a significant increase in propionate concentration and reduction in the ratio of A:P. These results suggest that *E. glabra* caused a shift in the ruminal microbial community, which

is reflected in the changes in the populations of the two major cellulolytic bacterial species examined (*R. albus*and *F. succinogenes*). The changes of population of *R. albus* and *F. succinogenes* suggest there was a shift in the dominant cellulolytic bacterial community. The *F. succinogenes* population was reduced immediately and remained significantly lower than control values throughout the experiment. In contrast, the *R. albus* population declined initially, before recovering after 11 days fermentation with *E. glabra*. This supports the suggestion of Goiri et al (2009) that a two-week incubation of dietary treatments in an open system is adequate to observe any adaptation of ruminal microbes. Cellulolytic microbes are major acetate and hydrogen producers. Fermentation towards acetate concentration is associated with more hydrogen released for CH_4 formation, while propionate concentration and methanogenesis are competitive pathways of hydrogen utilization (Wolin & Miller 1982; Hegarty & Gerdes 1999; Moss et al. 2000). The reduction of *F. succinogenes* and *R. albus* in this experiment supported the gradual decrease in acetate concentration by *E. glabra* treatments. In the EG25 treatment, after two weeks the *R. albus* population recovered and there was a corresponding increase in CH_4 and total gas production. This indicates that *E. glabra* is influencing the pathways of hydrogen utilisation but that there was some adaptation of the ruminal bacterial population over time.

From the propionate results, I can conclude that at least some of the modulating effect *E. glabra* has on CH_4 production is through indirect effects on the bacterial species involved in the utilization of hydrogen. However, in the current study, I only analysed three representative members of rumen bacterial species, one of which was not detected in any samples that were taken (*R. flavefaciens*), so it is difficult to draw stronger conclusions about the mechanism of action behind the effects of *E. glabra.* Further studies with pure cultures are needed to obtain a more complete understanding on the effect of *E. glabra* on specific and general effects on microbial ecology in the rumen and its mode of action.

4.5 Conclusion

Using a continuous fermentation system (Rusitec), I have demonstrated that the antimethanogenic effects of *E. glabra* are dose-related over three weeks and that reduction in CH_4 production appears to be through both direct and indirect effects on methanogens and other ruminal bacterial species. We cannot draw definitive conclusions about the mechanism of action of *E. glabra* in reducing CH_4 and more

detailed predictions about its effects on ruminal microbial ecology on the basis of an *in vitro* study is not wise. However, the lower amounts of *E. glabra* reduced CH_4 with only a slight reduction in other end products of fermentation and no effect on the disappearance of dry matter. In conclusion, feeding animals *E. glabra* at 150 g/kg of the diet would be safe and suitable for establishing whether it has the potential to reduce CH_4 production without affecting animal productivity.

Chapter 5 A diet containing *Eremophila glabra* reduces methane production in sheep

Abstract

Eremophila glabra Juss. (Scrophulariaceae) mitigates methane production when fermented *in vitro* for 21 days at a ratio of 15:85 in an oaten chaff/lupin mix, with minimum side effects on production of total gas and total volatile fatty acids. To confirm this finding *in vivo*, 1-year-old Merino × Suffolk wethers were offered one of two diets at 1.5 times their requirements for maintenance: control diet (1 kg oaten chaff/lupin mix) and *E. glabra* diet (150 g/kg *E. glabra* + 850 g/kg Control diet). The diets were fed for 30 days and feed digestibility, methane output and rumen fermentation were measured. Blood was sampled to confirm absence of general health problems in the sheep. Concentration of total and individual volatile fatty acids, ammonia and apparent nitrogen content were not affected by diet. Compared to the control, feeding *E. glabra* reduced methane emissions by 11.8% per gram of dry matter intake and 14.8% methane per gram of digested dry matter. This study shows that *E. glabra* changes fermentation in the sheep rumen without affecting intake or digestibility of the diet, suggesting it is a promising natural methane inhibitor. The potential of *E. glabra* in mitigating ruminant methane now needs to be assessed in on-farm research into grazing systems.

Key words: sheep, methane chamber, methane production, feed digestibility

5.1 Introduction

In the Mediterranean-type climatic regions in Australia, perennial native shrubs are fully adapted to drought and the low rainfall environment. If used as forage, such shrubs can provide alternative feed in the dry periods when traditional grass and legume pastures offer very little nutritional value. Incorporation of forage shrubs into farming system can thus support animal production and feed utilization in the low-medium rainfall zones in Australia, especially during the annual 'autumn feed gap' (Revell et al. 2008). There are major economic and environmental benefits of forage shrubs in a typical dry land farm in Western Australia (Monjardino et al. 2010).

Some woody perennial plants have been assessed for their ability to manipulate rumen fermentation and reduce methane (CH_4) production in batch culture fermentation,

including *Eremophila glabra* Juss. (Scrophulariaceae) (Durmic et al. 2010a). However, it is unlikely that such plants can be used as the sole diet for animals because of possible side-effects on rumen fermentation and an inability to provide sufficient edible biomass to support animal production. Therefore, I need to investigate the potential usage of *E. glabra* as one part of a mixed diet for ruminants.

In Chapter 4, the results from a Rusitec study (a continuous *in vitro* system) demonstrated that when *E. glabra* was mixed with oaten chaff/lupin at a 15 to 85 ratio, CH_4 production was reduced by up to 35%, and normal rumen microbial fermentation was sustained for up to 21 d. *E. glabra* seemed to alter rumen fermentation patterns, in particular by increasing propionate concentration and decreasing the acetate:propionate ratio (A:P) with an associated reduction in CH_4 production. However, there were also some negative effects of *E. glabra*, including limiting total gas production and inhibiting the cellulose degrader-*Fibrobacter succinogenes*.

It is difficult to extrapolate the effect from *in vitro* measurements to *in vivo* situations in ruminants (Flachowsky & Lebzien 2012). For example, the relationship between CH_4 measured *in vitro* and *in vivo* is usually very poor (R^2 = 0.26; Moss and Givens (1997)) and the total VFA concentration and neutral detergent fibre (NDF) digestibility in the Rusitec can also be lower than *in vivo* (Hristov et al. 2012). Nevertheless, I expected that sheep fed *E. glabra* as 15% of their diet could produce 35% less CH_4 than animals fed the same diet without *E. glabra* whilst maintaining productive performance.

5.2 Materials and methods

The experiment was conducted following the Australian National Health and Medical Research Council guidelines. The use of animals and the experimental protocol were approved by the Animal Ethics Committee of the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Livestock Industry, Floreat, Western Australia (Reference number: 1003).

5.2.1 Experimental design

Twenty four wethers (one year old, Merino × Suffolk, average initial live weight 39 ± 1 kg) were randomly assigned to two iso-energetic and iso-nitrogenous diets: 1) Control diet (n = 12): oaten chaff/lupin grain based diet; or 2) *E. glabra* (EG) diet (n = 12): 150 g/kg DM EG with 850 g/kg DM control diet. The chemical compositions of both diets

are shown in Table 5.1. The experiment lasted for 67 d: 32 d for all sheep to acclimatize to control diet and the animal house environment – it also ensured no other diets that sheep might fed prior to experimentation influenced measured parameters. This was followed by 4 d adaptation to EG diet, then followed by a further 30 d on control and EG diets. CH_4 production was measured on Day 30 and Day 31 followed by a rumen sample collection to analyse concentration of VFA and ammonia. Feed digestibility was measured one week before the CH_4 measurement. Animal performance was assessed as live weight change, feed digestibility and rumen fermentation outputs. Animal health was assessed by gross observation and by monitoring blood parameters.

Table 5.1. Chemical compositions of the experimental diets fed to sheep.

Diet	Control	EG
Ingredient composition		
Lupin (%)	17.5	15.0
Oaten hay (%)	78.5	66.0
E. glabra (%)	0.0	15.0
Mineral mix (%)	2.0	2.0
Molasses (%)	2.0	2.0
Analytical composition[a]		
Dry matter (DM, g/kg)	935	937
Neutral detergent fibre (NDF, g/kg DM)	253	244
Acid detergent fibre (ADF, g/kg DM)	239	242
Crude protein (g/kg DM)	111	112
EE (g/kg)	23.2	22.0
Metabolizable energy (ME, MJ/kg)	9.69	9.73

[a]Australian fodder industry association (AFIA) standard lab fodder analysis.

5.2.2 Diet preparation

E. glabra leaves were harvested from multiple plants (< 1year, approx. 1200), at the post-flowering stage in August 2011 at the University of Western Australia (UWA) Future Farm ("Ridgefield", Pingelly, Western Australia). Leaf samples were air-dried and kept at room temperature in sealed paper bags. Both diets were prepared as loose

mix and supplied by Glen Forrest Stockfeeders (Glen Forrest, Western Australia), one week prior to experimentation (Table 5.1).

5.2.3 Animal management and feeding

All sheep were housed in individual pens in the animal house at CSIRO, Floreat, Western Australia. All sheep had similar body weight that was within 1 kg difference. Therefore, they all received 1 kg/d of the diet, 1.5 times the maintenance requirement for metabolizable energy, and had free access to water (ARC 1980). The EG diet was introduced on Day 33. Sheep were gradually introduced to EG diet over 4 d, before receiving the full EG diet. Diets were offered once daily at 0830 h, with allowance for 10% refusal, and the feed intake was recorded every 24 h by subtracting feed residue from the amount of feed supplied. Body weight was measured every two weeks before the feeding and during the experiment. Sheep were adapted to the CH_4 chambers prior to the CH_4 measurement.

5.2.4 Rumen fluid sampling

After the CH_4 measurement, sheep were sent back to animal house and continually feeding on the same diet individually. Rumen fluid was sampled using a stomach tube 2 h after feeding. The sampling tube was washed after each sample was collected. The first 100 mL from each sample was discarded to minimize contamination by saliva. Approximately 50 mL of rumen fluid from the second sample was collected into sealed container and immediately placed onto ice. Thereafter, two 1 mL sub-samples were each mixed with 200 µL 1M NaOH or 2M HCl in separate eppendorf tubes (Sigma-Aldrich) and immediately stored at -20 ℃ to await VFA and ammonia analysis. VFA and ammonia were analyzed as described previously (Durmic et al. 2010a).

5.2.5 Feed digestibility

Sheep were fitted with a faecal harness 2 d before faecal collection to allow time for adaptation. After 23 d feeding on the control and EG diets, all faeces from each sheep were collected daily and weighed for 6 days. A sub-sample of the daily faecal output was retained (10%), weighed and oven-dried at 60 ℃ for three days to determine total dry faecal output. The apparent digestibility of dry matter (DMD) was calculated as follows:

Dry matter digestibility (DMD%) = 100*[1 – g DM faecal output]/g DM intake

5.2.6 CH_4 measurement

CH_4 measurement was carried out on Day 30 of feeding the EG and control diets using four open-circuit respiration chambers described by Klein and Wright (2006). Four sheep were measured in four individual respiration chambers each day, and CH_4 concentration was measured continuously for 23 h with one hour used to exchange sheep. CH_4 measurement in 24 sheep was completed over six consecutive days. The starting date of feeding the 12 sheep the EG diet was arranged over six consecutive days in order to keep each sheep on EG diet for exactly 30 d before CH_4 measurement.

To measure CH_4, sheep were brought in to the respiration chambers at 0930 h and were kept in the chamber for 23 h. The respiration chamber had enough room for sheep to turn around inside the chamber. Air was continuously drawn out by vacuum pumps and collected from the outlet of each chamber and, every 5 min, a sample was directly supplied to a gas chromatographer (Shimadzu GC-2010 Shimadzu Corporation, Kyoto, Japan), equipped with 2 flame ionisation detectors, 3.2 mm × 3.05 m stainless steel columns packed with molecular sieve 5A, 80/100 mesh (Alltech Associates Pty Ltd, Baulkham Hill, NSW), and 2 Valco valves fitted with 1.0 mL sample loops. The carrier gas was nitrogen at 400 kPa head pressure. The oven and detector temperatures were set isothermally at 150 ℃ and 300 ℃. Sample injection, data acquisition, and calculation of CH_4 peak areas were achieved by Shimadzu GC solution Chromatography Data System, Version 2.3 (Shimadzu Corporation, Kyoto, Japan). The gas chromatograms were calibrated twice each day (0900 and 1530 h) using 100 mg/L in a nitrogen Micromat-14 gas standard mix (GMT10341TC, Alltech Associates Pty Ltd, Baulkham Hill, NSW) and 10 mg/L CH_4 in a nitrogen standard mix (BOC Gas, Perth, WA). During CH_4 measurement, temperature, relative humidity, air pressure and carbon dioxide concentration in the chamber were monitored to ensure that test animals were comfortable and safe.

The feeding schedule in the CH_4 chamber was the same as in the pen and DM intake per individual sheep was measured, as described above. The 23 h CH_4 production was converted to 24 h CH_4 production, expressed as L/d, L/g DM intake and L/g digested DM.

5.2.7 Animal health

Animals were monitored daily for any gross abnormalities (reduced intake, alertness). In addition, jugular blood was collected into 10 ml lithium-heparin tubes (Becton, Dickinson and Company) for each sheep after the CH_4 measurement. Samples were centrifuged (2000×g for 10 min) and split into plasma and red blood cell fractions. The plasma was frozen at -20 ℃ and stored for analysis of liver function, kidney function, muscle function, and nutritional wellbeing (Olympus AU400, Auto Clinical Chemistry Analyser). The samples were analysed following the manufacturer's specifications at the Animal Production Laboratory, Department of Agriculture and Food Western Australia, South Perth, Western Australia. The detailed blood parameters are listed in Table 5.3.

5.2.8 Feed and faeces analysis

Samples (100 g) of both diets were dried at 60 ℃ for 48 h and ground (Glen Creston, Stanmore, England) to pass through a 1.0 mm sieve. Dried sub-samples of faeces from each animal were also ground and sieved. These samples were analysed for nitrogen content according to Standard Lab Fodder Analysis (AFIA).

5.2.9 Statistical analysis

The effects of diet on production of CH_4, concentration of VFA and ammonia, digestibility, nitrogen content, and blood chemical components (see Table 5.2 and Table 5.3 for full list of variables) were determined by separate one-way ANOVAs. Data were subjected to appropriate transformation to normalise the variance as required, but untransformed values are presented. All analyses were performed in GenStat (13th Edition, VSN International, 2010).

5.3 Results

5.3.1 CH_4 production and rumen metabolic parameters

The EG diet did not affect daily CH_4 production as CH_4 L/d, but there was 11.8% less CH_4 production as L/g DM intake and 14.8% less CH_4 production as L/g digested DM than sheep fed the Control diet ($P < 0.05$, Table 5.2). Substitutions of *E. glabra* in control diet did not affect concentration of total and individual VFA. Sheep fed the EG diet tended to produce a higher concentration of all individual VFA except butyrate.

There were no differences between dietary treatments in ammonia concentration or apparent nitrogen content.

5.3.2 Intake and feed digestibility

The EG diet did not affect DM intake, body weight gain or apparent digestibility (Table 5.2). Sheep fed EG diet consumed 16 g/d DM less daily than those on the control diet, with a 0.54 g/kg body weight gain per digested DM over the period of 30 days than those fed the control diet, the weight gain was not significant.

5.3.3 Animal health

All animals behaved normally and there was no change in body condition score during the experiment (Table 5.2). All blood analysis parameters in all sheep were within the normal range. Concentrations of GGT, GLDH, conjugated (direct) bilirubin, total bilirubin, creatinine, ALT, CK, BHB, total protein and albumin were not affected by EG diet (Table 5.3), but the EG diet reduced the average concentration of urea by 21.9% compared to Control diet ($P < 0.05$).

Table 5.2. Live weight, dry matter intake, CH_4 emissions and rumen fermentation parameters from wethers fed Control and EG diets.

	Control diet	EG diet	SEM	P
Initial live weight (kg)	39.5	40.2	0.54	0.37
Final live weight[c] (kg)	42.1	43.1	0.56	0.23
Dry matter intake[d] (g/d)	980	964	11.0	0.17
Dry matter digestibility (%)	0.612	0.607	0.01	0.74
Weight gain/digested DM (g/kg)	4.15	4.69	1.07	0.26
Condition score	2.83	2.90	0.03	0.15
CH_4 production (L/d)	10.9	10.1	0.39	0.17
CH_4 production[f] (L/g DM intake)	12.7[a]	11.2[b]	0.41	0.02
CH_4 production[g] (L/g digested DM)	21.0[a]	17.9[b]	0.83	0.02
Total VFA (mmol/L)	63.6	64.4	3.29	0.80
Acetate (mmol/L)	39.8	40.1	1.91	0.88
Propionate (mmol/L)	14.0	15.0	1.22	0.41
Butyrate (mmol/L)	7.10	6.90	0.61	0.82
Acetate: propionate	2.9	2.7	0.24	0.41
Apparent nitrogen content (%)	0.66	0.65	0.01	0.38
Ammonia (mg/L)	144	156	11.5	0.30

[a-b] Values in the same column with different superscript letters are significantly different at $P < 0.05$.
[c] Measured at the end of the experimental period.
[d] Dry matter intake over the last 30 days of the experimental period.
[e] Dry matter digestibility was measured from Day 23 to 29.
[f] CH_4 production was calculated according to DM intake of sheep in the chamber.
[g] CH_4 production was calculated DM intake in the chamber multiplied with DM digestibility.
SEM= standard error of means.

Table 5.3 Average concentration of blood chemical components and the standard reference ranges used to indicate liver, kidney and muscle function in sheep fed control and EG diets for 30 days.

Blood chemical components*	Reference range	Control diet	EG diet	SEM	P
Liver damage/ function indicator					
GGT (U/L)	23 – 67	43.4	43.5	4.66	0.99
GLDH (U/L)	< 20	15.5	19.3	4.83	0.44
Conjugated bilirubin (μmol/L)	< 15	0.17	0.39	0.18	0.24
Total bilirubin (μmol/L)	0-5	3.75	3.77	0.38	0.96
Kidney damage/ function indicator					
Urea (mmol/L)	3.3 – 8	6.08	4.75	0.40	0.03
Creatine (μmol/L)	50 – 150	89.2	88.9	4.38	0.96
Muscle damage indicators					
ALT (U/L)	< 30	15.2	17.9	1.43	0.06
CK (U/L)	< 500	178	128	35.4	0.17
Nutritional wellbeing					
BHB (mmol/L)	< 0.7	0.41	0.33	0.04	0.06
Total Protein (g/L)	60 – 75	65.2	64.4	1.03	0.45
Albumin (g/L)	28 – 34	31.1	31.2	0.54	0.98

*GGT: gamma-glutamyl transferase, GLDH: glutamate dehydrogenase, ALT: alanine aminotransferase, CK: creatine kinase, BHB: β-hydroxybutyrate.

SEM= standard error of means.

5.4 Discussion

The present study shows that inclusion of 15% *E. glabra* in a fibrous diet, the same level as that used in Rusitec study (Chapter 4), can reduce CH_4 production by more than 10% without interfering with animal productivity, health and performance. Importantly, sheep fed the EG diet had a slightly improved feed efficiency and tended to gain more body weight per unit of digested DM, compared to sheep fed on the control diet. The EG diet had a 0.8% lower DM digestibility than the control diet, suggesting that there was a reduction in energy loss from CH_4 output in sheep fed the EG diet, so more energy could be used for animal production. This aligns with recent findings that sheep gained weight and had lower CH_4 output per body weight gain during autumn when grazed on a shrub-based system (4 different shrub species) with annual pasture (Revell et al. 2013).

A reduction of CH_4 production in the rumen is associated with less energy loss from CH_4 and more energy consumed for animal production. Importantly, the efficiency of energy utilization in propionate by ruminants is much higher than acetate, for example, the efficiency for maintenance is 0.865 (maintenance) and 0.563 (fattening) for propionate, while it is 0.592 (maintenance) and 0.329 (fattening) for acetate (Blaxter 1962). This trend (the absolute values were not significantly different) towards improved efficiency of fermentation is reflected in the higher live weight gain in sheep fed on the EG diet. However, a longer feeding experiment is required to confirm whether these favorable trends in rumen fermentation persist and become significant. Similarly, when testing the potential of *E. glabra* to reduce the risk of animals developing acidosis, wheat was used to initiate acute acidosis and *E. glabra* was substituted at 200 g/kg. The concentration of VFA were not affected (Durmic et al. 2012). There is also evidence that the effectiveness of a supplement can depend on the nature of the base diet (eg, oaten chaff/lupin in this study). For example, supplementing linseed to cattle reduced total VFA production when they were fed barley silage but not when they were fed grass hay (Chung et al. 2011). Feeding *E. glabra* to sheep in this experiment produced favourable effects on live weight gain that were associated with favourable trends in the amount and types of VFA produced during fermentation in the rumen. These results confirm that it is safe to feed *E. glabra* to sheep and when it is used as a minor component of the diet (15%), it can improve the efficiency of production.

The fermentation characteristics of sheep fed *E. glabra* diet suggest that there was a direct inhibition on methanogens, rather than impaired cellulolytic activity. However, in the Rusitec study, *E. glabra* modulated the population of both methanogens and cellulolytic bacteria such as *Fibrobacter succinogenes* and *Ruminococcus albus* (Chapter 4). The differential effect on methanogens was also found when coconut oil and chestnut tannins were added into a diet for sheep (Liu et al. 2011). Both coconut oil and chestnut tannins reduced CH_4 production and the population of methanogens and protozoa. The population of *F. succinogenes* was decreased by coconut oil, whereas the populations of *R. flavefaciens* and *R. albus* were not changed by coconut oil and chestnut tannins and the molar proportion of individual VFA were not affected. Clearly, further studies of rumen microbiology are required to determine if populations of major cellulolytic bacteria are inhibited *in vivo* in sheep fed *E. glabra*. It is only possible to speculate whether any of rumen bacteria adapted to the *E. glabra* diet during the present study based on the VFA values collected at the end of the feeding period, because any changes of total VFA production may disappear after over 20 days feeding (Zhang et al. 2011).

The maintenance of total VFA concentration and acetate concentration indicates that *E. glabra* did not inhibit fibre digestion. Sheep fed on EG diet had a lower A:P ratio compared with sheep fed on control diet, suggesting that less hydrogen was available for methanogenesis. There is a typical inverse correlation between the production of CH_4 and propionate (Wolin 1960), which has been reported for several bioactive plants such as, *Acacia mearnsii* (Carulla et al. 2005), *Rheum officinale* (Garcí a-Gonzá lez et al. 2012) and incubating *E. glabra* in Rusitec (Chapter 4). However, an increase in propionate concentration was not detected in the rumen samples collected from sheep in this experiment. There are two possible explanations. First, I used small numbers of sheep, and it is possible that using more sheep may have improved the ability to detect significant differences in propionate concentration in sheep fed *E. glabra* diet compared to the control diet. Second, hydrogen that is used by methanogenesis was not diverted to produce propionate in this study. However, it would be necessary to complete a full microbial ecology or metagenomic study of the microbes in the rumen of animals consuming *E. glabra* to help clarify and make more specific conclusions about hydrogen utilization.

Compared to the 35% CH_4 reduction in the Rusitec (Chapter 4), there was only a 14.7% reduction in CH_4 per digested DM observed *in vivo* at same substitution level. There are two reasons to explain this. The dilution rate in the rumen *in vivo* is higher than in the Rusitec, with constant rumination and flushing by saliva. It is possible that *E. glabra* may not have remained in the rumen for long and therefore been as effective in the rumen as it was in the fermentation vessel in the Rusitec. It is also possible that methanogens and/or protozoa are more sensitive to *E. glabra in vitro*, as this is an artificial system (Rusitec). Clearly the Rusitec fermentation overestimated the capacity of *E. glabra* to mitigate CH_4, and it is possible that a higher substitution level of *E. glabra* may reduce CH_4 to a similar level as in the Rusitec. However, even though it may be possible to achieve a greater reduction in CH_4, I have established that there is still a significant reduction in CH_4 when *E. glabra* is a minor component of the diet.

In addition to the effect of *E. glabra* on CH_4 abatement, I evaluated palatability and digestibility of *E. glabra* in sheep for the first time, an important issue that needs to be considered before substituting bioactive plants in ruminant diets. During the 4-day period of adaptation to the *E. glabra* diet, sheep tended to leave *E. glabra* leaves aside for the first two days, but they eventually adapted and consumed the *E. glabra* diet. However, after experimentation, anecdotal evidence suggested that when sheep had both control and EG diet to choose in the paddock, most of the sheep preferred the control diet. By comparison, sheep which trained to *E. glabra* diet finished the EG diet. Animals can select or reject a feed by the food's odour (Forbes 1998). Cow and horse preferences to oaten hay were strongly related to the abundance of two volatile compounds emitted from the hay (Pain et al. 2005; Pain 2008).It is possible that the aroma from *E. glabra* leaves may deter sheep as well. This suggests that *E. glabra* might not be a preferred feed source for sheep to graze freely, but, during the seasonal 'feed gap', when there is little choice, it seems likely that they will forage on *E. glabra*.

5.5 Conclusion

Sheep fed a fibrous diet with 15% substitution of *E. glabra* showed reduced CH_4 production, in line with our previous findings with the Rusitec fermentation system. After training the sheep to eat the diet containing *E. glabra*, their daily feed intake, body weight, and rumen fermentation were not affected. These results suggest that *E. glabra* is a practical feed source for sheep that could reduce CH_4 emissions in grazing systems, and could help reduce agricultural greenhouse gas emissions. I now have a better

understanding of the use of *E. glabra* as an animal feed, and confirmed its potential as a forage shrub, particularly as a contributing source of feed during the dry season in Mediterranean environments.

Chapter 6 Extraction and isolation of antimethanogenic fractions from *Eremophila glabra*

Abstract

Eremophila glabra Juss. (Scrophulariaceae) – an Australian native shrub rich in secondary compounds – has the potential to mitigate ruminant methane. This study aimed to determine the active fractions of *E. glabra* responsible for low methanogenesis. A stepwise approach was used to generate extracts of *E. glabra* and chromatographic fractions, which were then assessed in batch culture fermentation for 24 h for antimethanogenic activity. *E. glabra* was first examined in the presence of a tannin binder to show that tannins were not responsible for the antimethanogenic effect of *E. glabra*. The second step was to obtain crude extracts using five solvents to determine the most effective solvent. Methanol/chloroform (1:1, v/v) was found to produce the most prominent antimethanogenic extract, as well as a good biomass return. This extract was separated to 11 fractions eluted in a series of solvents by silica gel chromatography. Among those fractions, only two, eluted with ethyl acetate/hexane (1:4, v/v) and methanol/ethyl acetate (1:1, v/v), showed antimethanogenic effect. The ethyl acetate/hexane fraction contained hydrocarbon compounds and therefore prevented further separation. The methanol/ethyl acetate fraction was subjected to high performance liquid chromatography to provide 14 fractions, but none of them reduced methane production in batch culture fermentation. The results from this study show that fractions of *E. glabra* have antimethanogenic bioactivity, but their identity remains to be classified.

Key words: fraction, isolation, silica gel column chromatograph, high performance liquid chromatography

6.1 Introduction

Plants with antimicrobial properties can modulate rumen fermentation pattern (Wallace et al. 2002) and inhibit methanogenesis (Busquet et al. 2005; Patra et al. 2006; Bodas et al. 2008; García-González et al. 2008a; Durmic et al. 2010a). These bioactive plants and their extracts (phytochemicals) can be effective in mitigating methane (CH_4) emissions as they alter the fermentation by affecting rumen microbes (Busquet et al. 2006; Patra et al. 2006; Kamra et al. 2008; Geraci et al. 2012; Goel & Makkar 2012; Cieslak et al.

2013). Plants, such as, *Eremophlia glabra* Juss. (Scrophulariaceae) (Durmic et al. 2010a), *Rheum nobile*, *Salix caprea* (Bodas et al. 2008), *Yucca schidigera* (Goel & Makkar 2012), *Albizia rhizonse*, *Sapindus saponaria* (Soliva et al. 2008), *Terminalia chebula* (Patra et al. 2006), chestnut and coconut (Liu et al. 2011) and *Vaccinium vitis idaea* (Cieslak et al. 2012) have been tested for their potential to reduce CH_4 emissions from the rumen. The main phytochemicals thought to be responsible for reducing methanogenesis are tannins, saponins and essential oils, and they act by either directly affecting the methanogen population or by indirectly affecting fibre digestion (Hess et al. 2003; Hart et al. 2008; Kamra et al. 2008; Patra & Saxena 2010).

E. glabra has been found to be effective in reducing enteric CH_4 production from sheep (Chapter 5). *Eremophila* (Myoporaceae) species generally grow in arid or desert areas (Richmond & Ghisalberti 1994) and they are characterised as tolerant to drought, fire, frost and grazing (Frazier 1965; Richmond 1993). They are highly valued for medicinal usage and for their antimicrobial action, mostly because of their aromatic smell (Ghisalberti 1994a; Ghisalberti 1994b). *E. glabra* is considered as a traditional Australian Aboriginal medicinal plant (O'Connell et al. 1983; Smith 1991; Aboriginal Communities of the Northern Territory of Australia & Barr 1993) and extracts of *E. glabra* have been found to be effective against *Campylobacter jejuni* (Kurekci et al. 2012), and against sheep gastrointestinal worms (Kotze et al. 2009).

E. glabra leaves are coated with a resin that is rich in secondary compounds that might be responsible for its antibacterial effects (Ghisalberti 1995). It includes a wide variety of diterpenes and sesquiterpenes (Ghisalberti 1995) and can be extracted with solvents such as ether or acetone (Ghisalberti 1994b). A number of diterpenes from the leaves and terminal branches of *Eremophila* have been isolated (Forster et al. 1986), including the serrulatane diterpenes, phenolic compounds that are likely to have antimicrobial effects in the rumen. In addition, one of the diterpenes extracted from *E. glabra* was reported to inhibit the growth of *Streptococcus bovis*, a gram-positive amylolytic rumen bacteria (Hutton et al. 2012), which converts carbohydrate to lactic acid. In a preliminary study, a crude ethanolic extract of this plant demonstrated antimethanogenic properties (Durmic et al. 2013). Therefore, to test the hypothesis that the extractable components from *E. glabra* will reduce CH_4 production, organic solvents and chromatography techniques were used to isolate fractions of *E. glabra* and tested them for antimethanogenic activity in batch culture fermentation.

6.2 Materials and methods

6.2.1 Experimental design

The initial challenge was to optimise the extraction of fractions and then purify compounds using chromatography techniques. A stepwise approach was used to isolate fractions of *E. glabra* that could then be mixed with oaten chaff in batch culture to examine their antimethanogenic activity. Oaten chaff was chosen as the substrate in the assay because it has relatively high methanogenic potential and does not contain any known phytochemicals (Durmic et al. 2010a). It has also been used as a base substrate throughout the experimental work in this thesis.

E. glabra was first incubated with or without polyethylene glycol (PEG), a tannin-inactivating agent, in batch culture, to test whether tannin activity in the leaves was responsible for any antimethanogenic effects (Makkar et al. 1995).

E. glabra was extracted with five alternative solvents to obtain crude extracts. Two solvents were selected based on the batch culture results. Further selection of the solvent was determined based on biomass return of the extraction and the most effective solvent was chosen based on the combination of the strength of the antimethanogenic effect and the amount of biomass return.

A crude extract with the preferred solvent was then separated by silica gel column chromatography and each of the resulting fractions was tested in batch culture. The prominent antimethanogenic fraction was then subjected to high performance liquid chromatography (HPLC) for identification of the fraction. The resulting isolates were tested in batch culture.

6.2.2 Plant material

E. glabra (accession number SA 45599) were harvested from multiple plants grown at an experimental site under the control of the South Australian Research and Development Institute (SARDI, Waite Institute, South Australia) at reproductive stage (< 1year old) in February 2008. Leaves were collected, freeze dried, sealed in an evacuated bag and stored at room temperature. Samples were ground through 1.0 mm screen (Glen Creston, Stanmore, England) and used for extraction and batch culture fermentation.

6.2.3 In vitro batch culture fermentation assay

The inoculum for batch culture was collected from three fistulated Merino sheep 2 h after the morning feeding. The sheep had been fed on a standard oaten chaff/lupin diet (oaten chaff: lupin: minerals = 40:10:1) *ad libitum* for two weeks before sample collection, and had unlimited access to water. Rumen fluid was collected using a vacuum pump, pooled and strained through cheesecloth, then transferred to an anaerobic chamber and buffered to pH 7.2 (McDougall 1948).

In vitro batch culture was used following the procedures described by Durmic et al. (2010a). Briefly, one day prior to the experiment, 0.1 g oaten chaff was weighed into individual Bellco tubes and kept in an anaerobic chamber (Coy Vinyl Anaerobic Chamber; Coy Laboratory Products Inc., USA) supplied with 800 mL/L N_2, 100mL/L CO_2, and 100 mL/L H_2 to expel any oxygen from the bottle. On the experimental day, 10 mL buffered rumen inoculum was dispensed into the prepared Bellco tubes and treatments were added. Each tube was sealed with a rubber stopper and crimped with an aluminum seal and incubated at 39 ℃ with constant shaking at 50 rpm. After 24 h incubation, gas samples were collected for measurement of total gas production and CH_4 concentration, as described by Durmic et al. (2010a). Each treatment had three replicates.

Both crude extracts and plant fractions from 0.5 g *E. glabra* were prepared 3 h before *in vitro* fermentation. Each crude extract and fraction was dissolved completely in 0.5 mL 100% ethanol (EtOH) and kept in a sealed glass container at room temperature until experimentation. Dissolved fractions or crude extracts (0.1 mL each, equivalent to 0.1 g plant material) were added into the Bellco tubes and mixed with 10 mL buffered rumen inoculum. The production of CH_4 and total gas was measured at the end of fermentation.

For the PEG test (refer to 6.2.4), the control was 0.1 g oaten chaff. When ethanol was used in the assay, the control included 0.1 g oaten chaff with 0.1 mL EtOH to account for the potential effects of EtOH on fermentation.

6.2.4 Polyethylene glycol (PEG) test for tannin

PEG was incubated with 0.1 g *E. glabra* in batch culture fermentation to eliminate any potential tannin effects on rumen fermentation. Briefly, treatments were 0.1 g *E. glabra*,

with or without 0.2 g PEG (Goel et al. 2008). PEG was first dissolved in 10 mL buffered rumen inoculum prior to mixing with plant material. Total gas and CH_4 production was compared between *E. glabra* and *E. glabra* +PEG.

6.2.5 Crude extracts

Plant material (0.5 g) was dissolved respectively in 5 mL solvents, water, ethanol, methanol (MeOH), acetone, or MeOH/chloroform (1:1, v/v), and macerated for 12 h shaking on rotator at room temperature. Thereafter, the plant material plus solvent was centrifuged at 13,000 rpm for 10 min and the supernatants were collected and filtered into eppendorf tubes. The remaining pellet of plant material was extracted again with the same volume of solvent, and the second extract was pooled with the first. The fibrous residue was discarded. The pooled extract was then passed through a sterile filter (0.22 µM) and evaporated under vacuum at 40 ℃. The crude extracts were stored at -20°C until batch culture fermentation assay (refer to 6.2.3).

Based on the tannin test results (refer to 6.2.4), a general plant extraction protocol was prepared following the procedure described in Hutton et al. (2012). Two solvents, MeOH/chloroform (1:1. v/v) and acetone, were selected based on the results from the batch culture fermentation of crude extracts (see above). Briefly, ground leaves of *E. glabra* (0.46 g) were added to 20 mL of one of the solvents, covered and stirred for 1 h. The samples were filtered (Whatman No.1) and the extract was dried under vacuum in a water bath with temperature not exceeding 40 ℃. The weight of extract was recorded to calculate the biomass return of the extraction.

6.2.6 Silica gel column chromatography on crude extract

The following plant extraction work was carried out in collaboration with Dr Gavin Flematti at the School of Chemistry and Biochemistry (The University of Western Australia). A crude extract was carried out with 5 g of plant material in 150 mL MeOH/chloroform (1:1, v/v) and stirred for 1 h. The mixture was filtered (Whatman No.1, 24 cm), evaporated to dryness using a rotary evaporator, then dissolved in 5 mL methanol/chloroform and applied to silica gel chromatography (Merck silica gel 60).

The silica gel column was equilibrated with hexane before the crude extract was loaded. After loading the crude extract, the column was eluted with a series of solvents (60 mL each) in order of 100% hexane, 20% ethyl acetate (EtOAc)/hexane (×3), 40%

EtOAc/hexane, 60% EtOAc/hexane, 80% EtOAc/hexane, 100% EtOAc, 10% MeOH/EtOAc, 50% MeOH/EtOAc and finally 100% MeOH. The eluate corresponding to each solvent was collected and 11 fractions were generated. The eluate was concentrated under vacuum and dried under a stream of nitrogen (N_2) gas. The dried eluate was stored in a sealed glass container at 0 ℃. A subset of fractions (ca. 10%, individual fraction equals to 0.5 g dry matter of plant material) were taken and tested for antimethanogenic activity in batch culture.

6.2.7 HPLC isolation of the selected fractions from silica gel chromatography

Two fractions from the silica gel chromatography with antimethanogenic effects were subjected to HPLC in a system equipped with a multiple wavelength detector (Hewlett Packard, Model 1050, USA). A 250×10 mm Apollo C_{18}, 5 μm, reverse-phase column (Grace-Davison) was used. The eluate produced with 100% EtOAc was found to contain mainly hydrocarbons so it was no longer investigated. The dried eluate produced with 50% MeOH/EtOAc (refer to 6.2.6) was reconstituted in 5 mL acetonitrile and 100 μL (equivalent to the material from 0.5 g of the plant material) was injected into the HPLC column. The mobile phase contained 35% acetonitrile and 0.1% acetic acid in water at a flow rate of 4 mL/min and 4 mL fractions were collected at 1-min intervals for up to 40 min. Fractions were composed according to the signals of the detector at 220, 254 and 520 nm that indicated presence of compounds in the eluate. A total of 14 fractions were generated. They were concentrated under vacuum, dried under a stream of N_2 gas, and stored in a sealed glass container at 0 ℃ before batch culture fermentation.

6.2.8 Statistical analysis

To test for differences in total gas production and CH_4 production of *E. glabra* with or without PEG, the data were subjected to one-way ANOVA. The differences in total gas production and CH_4 production from the crude extracts, silica gel fractions and HPLC fractions, compared with controls, were also subjected to one-way ANOVA. When the ANOVA was significant, means were separated using Fisher's least significant difference (LSD, $\alpha = 0.05$). All statistical analyses were carried out using GenStat (13th Edition, VSN International, 2010).

6.3 Results

6.3.1 PEG test for effect of tannin

Incubation of *E. glabra* with PEG reduced total gas production, but did not affect CH_4 production (Table 6.1). Both *E. glabra* and *E. glabra*+PEG treatments produced less CH_4 than the control ($P < 0.05$).

Table 6.1 The production of total gas and CH_4 from batch culture fermentation of *E. glabra* with or without polyethylene glycol (PEG).

Treatments	Total gas production (mL/g DMi*)	CH_4 production (mL/g DMi)	CH_4 concentration (mL/100mL)
Control	389[c]	53.8[b]	13.9[b]
E. glabra	380[b]	26.0[a]	6.84[a]
E. glabra + PEG	362[a]	29.0[a]	7.95[a]
SEM	1.70	0.88	0.37

*DMi =dry matter incubated.
SEM = standard error of means.
[a-c] Values in the same column with different superscripts are significantly different (LSD, $P < 0.05$).

6.3.2 Crude extracts

Except for the water extract, all crude extracts of *E. glabra* had lower CH_4 production and total gas production compared to the control ($P < 0.05$, Table 6.2). Crude extracts of *E. glabra* from MeOH/chloroform (1:1, v/v) had the lowest CH_4 concentration and CH_4 production (more than 90% inhibition) compared to the control ($P < 0.05$).

The biomass return was 0.12 g (26%, by mass of plant material) for MeOH/chloroform (1:1, v/v) and 0.09 g (20%) for acetone. HPLC analysis showed that MeOH/chloroform (1:1, v/v) extraction contained the most diverse spread of compounds (chromatography not shown) and was chosen as the preferred solvent for crude extraction.

Table 6.2 The production of total gas and CH_4 in the presence of crude extracts of *E.glabra* using various solvents in batch culture.

Treatments	Total gas production (mL/g DMi*)	CH_4 production (mL/g DMi)	CH_4 concentration (mL/100mL)
Control	362^{d}	52.0^{c}	14.4^{c}
MeOH extract	335^{c}	37.0^{b}	11.0^{b}
Water extract	379^{e}	51.4^{c}	13.6^{c}
EtOH extract	320^{bc}	31.1^{b}	9.71^{b}
Acetone extract	307^{ab}	34.6^{b}	11.3^{b}
MeOH/chloroform extract	298^{a}	4.1^{a}	1.37^{a}
SEM	1.80	1.12	0.31

*DMi =dry matter incubated.
SEM = standard error of means.
$^{a-d}$Values in the same column with different superscripts are significantly different (LSD, $P < 0.05$).

6.3.3 Silica gel chromatographic fractions

A total of 11 fractions were generated from silica gel column chromatography of the MeOH/chloroform extract (Table 6.3). Fractions eluting with 20% EtOAc/hexane, 40% EtOAc/ hexane, 50% MeOH/ EtOAc and 100% MeOH had lower total gas production compared to control ($P < 0.05$). CH_4 concentrations were the lowest in the fractions from 50% MeOH/EtOAc and 20% EtOAc/hexane, being reduced by 51% and 48% ($P < 0.05$). In contrast, fractions eluted with 100% EtOAc and 10% MeOH/EtOAc increased CH_4 yield ($P < 0.05$).

Table 6.3 The total gas and CH_4 production of silica gel chromatographic fractions of MeOH/chloroform extract of *E. glabra* in batch culture.

Eluting fractions	Total gas production (mL/g DMi*)	CH_4 production (mL/g DMi)	CH_4 concentration (mL/100mL)
Control	357^{de}	50.5^{c}	14^{bc}
100% Hexane	348^{cde}	45.6^{bc}	13.1^{bc}
20% EtOAc/ hexane (1)	312^{a}	22.9^{a}	7.3^{a}
20% EtOAc/ hexane (2)	339^{bcd}	44.3^{bc}	13.1^{bc}
20% EtOAc/ hexane (3)	344^{cde}	43.2^{bc}	12.6^{cd}
40% EtOAc / hexane	329^{abc}	36.1^{ab}	11.0^{ab}
60% EtOAc / hexane	346^{cde}	44.5^{bc}	12.9^{bc}
80% EtOAc / hexane	352^{cde}	46.9^{bc}	13.3^{bc}
100% EtOAc	363^{e}	54.7^{c}	15.1^{c}
10% MeOH / EtOAc	361^{de}	54.1^{c}	15.0^{c}
50% MeOH / EtOAc	318^{ab}	22.0^{a}	6.9^{a}
100% MeOH	332^{abc}	44.4^{bc}	13.4^{bc}
SEM	3.49	2.19	0.6

*DMi =dry matter incubated.
SEM = standard error of means.
$^{a-e}$ Values in the same column with different superscripts are significantly different (LSD, $P < 0.05$).

6.3.4 HPLC fractions

A total of 14 fractions were generated from the 50% MeOH/EtOAc eluate of silica gel chromatography (Table 6.4). Compared to the control, there was no significant change in total gas production in any isolated fraction, while all fractions increased CH_4 production ($P < 0.05$).

Table 6.4 The total gas and CH_4 production of HPLC fractions of the 50% MeOH/EtOAc eluate from silica gel chromatography in batch culture.

Fractions	Total gas production (mL/g DMi*)	CH_4 production (mL/g DMi)	CH_4 concentration (mL/100mL)
Control	404	41.6^{a}	10.3^{ab}
1	408	54.0^{bcd}	14.2^{bc}
2	406	53.8^{bcd}	13.3^{abc}
3	404	54.6^{bcd}	13.5^{abc}
4	401	53.9^{bcd}	13.4^{abc}
5	410	54.4^{bcd}	13.3^{abc}
6	401	52.9^{bc}	13.2^{abc}
7	400	54.5^{bcd}	13.6^{abc}
8	400	53.8^{bcd}	13.4^{abc}
9	401	49.8^{b}	9.7^{a}
10	399	51.9^{bc}	13.0^{abc}
11	404	54.8^{bcd}	15.1^{c}
12	408	59.2^{d}	14.5^{bc}
13	410	56.5^{cd}	13.8^{abc}
14	406	55.5^{cd}	13.7^{abc}
SEM	2.08	0.81	0.71

*DMi =dry matter incubated.
SEM = standard error of means.
[a-d] Values in the same column with different superscripts are significantly different (LSD, $P < 0.05$).

6.4 Discussion

E. glabra contains extractable components with antimethanogenic activity, reducing CH_4 production by up to 92.1%, which supports other results where ethanolic extracts were observed to reduce CH_4 production (Durmic et al. 2013). The small reduction in total gas production shows that extracts from silica gel chromatography had a slight, but general inhibitory effect on overall fermentation that is consistent with the outcome of fermenting raw *E. glabra* leaves (Chapter 3 and 4). The combination of MeOH/chloroform (1:1, v/v) extraction with silica gel chromatographic isolation seems to be an effective approach for isolating of unknown extracts/fractions with potential antimethanogenic effect from *E. glabra*.

The most surprising result was that none of the 14 HPLC fractions isolated from the most active fraction from silica gel chromatography showed any antimethanogenic activity. The likely explanation is that the activity was lost in the isolation process, or that there was degradation of effective compound(s) during or before the HPLC isolation. It is also possible that an inadequate dose was applied. Each fraction tested was theoretically equal to the concentration of active compounds in 0.1 g of plant material. In future work, it would be useful to test a higher inclusion level of plant fractions to see whether an adequate dose of active fractions/compounds can be achieved. Another possibility could be that the antimethanogenic effect found in the most active silica gel chromatographic fraction was the result of synergistic interactions among some of 14 compounds isolated by HPLC, so future work should also test fractions in combination.

This experiment did not use sophisticated methodology for identifying compounds in the most active fraction from silica gel chromatography because the activity could not be detected in the subsequent fractionation, so potential compounds with possible antimethanogenic effects are only mentioned briefly here. From the PEG test, it is clear that the active components responsible for CH_4 reduction do not belong to the tannin class of secondary compounds. Serrulatane compounds are major compounds that have been identified previously in *E. glabra* and can inhibit lactate fermentation *in vitro* (Hutton et al. 2012), but these compounds did not exist in the most active fraction from silica gel chromatography. In future work, HPLC connected with mass spectrometry will be used for the identification of these compounds, so their chemical structures can

be determined and a better understanding of the potential mechanisms of antimethanogenic activity can be achieved.

When compared to the activity of whole plant material, the *E. glabra* fractions tested here generally had much smaller effects on methanogenesis. Similarly, Goel et al. (2008) found that the reduction in CH_4 output with water extract and MeOH/water extract of *Carduus* was not equivalent to that from whole *Carduus* plant material. Obtaining a practical amount of extract requires a large quantity of plant material and this limits the practical use of any active fractions that are identified if the selected plant species has low biomass. *E. glabra* is not a high biomass plant and so the pursuit of purifying and identifying active compounds will be important for understanding the mechanisms through which *E. glabra* reduces CH_4 production and as a marker for the presence of antimethanogenic activity. However, unless the compound identified can be already exists and can be purchased 'off the shelf' and/or be readily synthesised, incorporating *E. glabra* into grazing systems remains the best practical option for using *E. glabra* to reduce CH_4 production as well as gain a range of other benefits associated with shrub based systems.

6.5 Conclusion

Fractions that can be extracted from *E. glabra* can reduce CH_4 production, offering great opportunity to identify and select effective CH_4 inhibitors. It also provides information about the range and types of compounds that inhibit CH_4, which is beneficial because it increases the opportunities to achieve sustained reductions in CH_4 production. Identifying active fractions from *E. glabra* may also help us target plants that contain similar compounds and therefore be more efficient at identifying plants that are potential CH_4 inhibitors. However, the antimethanogenic activity seems to become more difficult to detect during the latter steps of isolation and purification of the active fraction. There could be a synergistic effect among fractions of *E. glabra* that contribute to low methanogenesis, or there could be loss and degradation so that the supplementation level was not effective enough to reduce CH_4. Future work should be focused on testing higher supplementation levels and combinations of fractions.

Chapter 7 General Discussion

In this thesis, I expected that the Australian native plant-*Eremophila glabra* could reduce ruminant methane (CH_4) production and could be fed to sheep without affecting animal production and health. The results from all experiments described in this thesis support the hypothesis that *E. glabra* inhibits CH_4 and the results from the *in vivo* experiment (Chapter 6) confirm that it can reduce CH_4 without having a detrimental effect on production parameters and animal health. There are three major findings reported in this thesis: (1) *E. glabra* has the potential to provide a valuable component of the feed base in novel grazing systems based on mixed forages, because it reduces CH_4 production without adversely affecting animal production and health when included at only 15% of the diet. This is particularly relevant to the current grazing systems in Mediterranean climates, where there is a gap in the feed supply during summer and autumn and native perennial forage shrubs can fill that gap; (2) *E. glabra* inhibits CH_4 by directly reducing the methanogen population, as well as by modulating chemical pathways of hydrogen release and utilization in the ruminal fermentation; (3) it is possible to extract fractions from *E. glabra* that have the antimethanogenic effect. Further research is required to specify the mechanisms of action, purify and identify the specific compounds responsible, but this information could be used to develop feed additives for mitigating CH_4 emissions from ruminants. These results extend our understanding on the benefits of incorporating *E. glabra* into grazing systems for ruminant livestock (sheep).

In the following discussion, I will highlight the role of *E. glabra* in mitigating ruminant CH_4 as well as comment on the steps that should be taken to minimize the potential side effect of *E. glabra.* The value of using continuous *in vitro* fermentation (Rusitec) before conducting animal experiment is also discussed, followed by a concluding remark about the work presented in this thesis.

The results from the *in vivo* experiment in this thesis are exciting because they provide evidence that *E. glabra* can be consumed in relatively small quantities and reduce CH_4 emissions from sheep without affecting their productivity and health. *E. glabra* has been one of the Australian native shrubs considered within the Future Farm Industry CRC 'Enrich' research project as having the potential to form part of a novel grazing system based on perennial shrubs (Revell et al. 2008; Durmic et al. 2010b; Revell et al. 2013). The main target of the 'Enrich' concept has been the low to medium rainfall zones of

Australia with Mediterranean climates, a feature of which is hot, dry summer and autumn periods where the amount and quality of feed is low because the current systems are based on annual, introduced, pasture species. Producers have to provide supplementary feed to fill this 'feed gap' in the summer and autumn to maintain livestock, which is expensive. The native perennial shrubs offer an alternative to supplementary feeding because they are drought tolerant and provide green feed throughout the year (Revell et al. 2008; Ben Salem et al. 2010; Norman et al. 2010; Revell et al. 2013). The economic value of incorporating native perennial shrubs into these systems has been modeled, which showed that planting perennial shrubs on up to 20% of the marginal land on farms improves profitability and the benefit is largely due to the reduction in the amount of supplementary feed producer needs to provide (Monjardino et al. 2010).

The basic principle behind 'Enrich' project is that Australian native plants can be a useful addition to pasture systems provided they are considered as part of a mixture rather than as a single species offering a 'silver bullet' solution. The usefulness of different shrub species within the mix depends on how they are valued and not all of the species need to provide nutritional value. For example, the value of having a species in the mixture of shrubs could be due to its ability to provide shade and shelter, reduce erosion, use water more efficiently within the soil profile, provide edible biomass and/or bioactive secondary compounds that affect animal and gut health (eg. anthelmintic, antimethanogenic and antimicrobial bioactivity) (Revell et al. 2008; Kotze et al. 2009; Durmic et al. 2010a). *E. glabra* does not produce large amounts of biomass, but it has good nutritive value properties and is grazed by livestock (Revell et al. 2013). However, the main value of *E. glabra* in the 'Enrich' system is its antimethanogenic bioactivity (Durmic et al. 2010a). Since *E. glabra* is a relatively small shrub that does not produce a lot of biomass, it will only ever contribute a small amount to the total diet of an animal grazing the shrub-based system. One of the key questions has been whether *E. glabra* could be consumed as a relatively minor component of a mixed diet and reduce CH_4 emissions without reducing animal productivity. The results from the *in vivo* experiment demonstrated that sheep fed *E. glabra* as a small proportion of their diet (15%) produce less CH_4 without reducing their intake or losing weight provide the first evidence that it can.

There is global interest in understanding how rumen fermentation can be manipulated to reduce CH_4 and improve the efficiency of utilization of feed. In principle, reducing the methanogen population and/or their activity directly or changing overall hydrogen availability in the fermentation system indirectly by affecting other ruminal microorganisms (eg. cellulolytic species) should lead to a reduction in CH_4 formation (Chaucheyras-Durand et al. 2010; Martin et al. 2010). *E. glabra* clearly has some general antimicrobial properties, because it severely inhibits fermentation when it is used as the sole substrate for fermentation *in vitro* (Durmic et al. 2010a). However, results from the *in vitro* studies (Chapter 3) suggest that *E. glabra* can have beneficial effects on fermentation and the negative impact on rumen fermentation can be moderated when plant is mixed with other substrate at lower substitution level. This study also provided evidence that the reduction in CH_4 by *E. glabra* is likely due to both direct and indirect effects on the methanogens (Chapters 3 and 4). The changes in VFA profiles when *E. glabra* was used as a substrate indicate that part of the reduction in CH_4 production is due to a reduction in hydrogen availability. Hydrogen availability is affected by the different pathways of carbohydrate degradation to VFA. For example, the formation of propionate from pyruvate sequestrates hydrogen, whereas production of acetate from pyruvate releases hydrogen (Moss et al. 2000; Janssen 2010). Therefore, the ratio of acetate to propionate (A:P) is associated with CH_4 production and as such is used as an indicator of CH_4 reduction. The expectation is that there will be an inverse relationship between CH_4 and propionate production, because of the competition for hydrogen utilization for these two pathways. This was reflected in the *in vitro* studies, where the reduction in CH_4 production was associated with a decrease in A:P ratios, because of the higher amount of propionate produced when *E. glabra* was used as a substrate (Chapter 3 and 4). This inverse relationship between CH_4 and propionate production has been reported for other studies involving bioactive plants (Lila et al. 2003; Garcá-González et al. 2008b; Holtshausen et al. 2009). However, similar changes in VFA production in the rumen did not occur *in vivo*, when sheep were fed *E. glabra* at 15% of their diet in the animal house (Chapter 5). *In vitro* experiments have limitations and should only ever be used as a guide to what should be tested, or may occur, *in vivo*. One limitation of *in vitro* studies is that they are less dynamic and less diverse in terms of their microbial ecology compared to what occurs *in vivo*. It is likely that the more complex microbial ecology and dynamic system in the rumen of animals promoted or expressed more hydrogen utilization pathways than *in vitro* studies, but it is clear that at

low levels *E. glabra* can have positive effects on rumen fermentation both *in vitro* and *in vivo*. This is important because it demonstrates that plants that would otherwise be dismissed as potentially useful pasture species, may in fact be valuable provided they are considered as part of a mixed pasture. Further research is required to determine how these chemical and biochemical pathways are altered by *E. glabra*, so that modulation of rumen fermentation can be achieved more precisely for both CH_4 mitigation and animal production.

The microbial profiling undertaken in this study was limited to methanogen populations and some cellulolytic bacterial species. The results were informative and also support the conclusion that *E. glabra* has both a direct effect on the methanogen population as well as an indirect effect on methanogenesis, through its effects on cellulolytic species in the rumen (Chapter 4). There is preliminary evidence that crude extracts from *E. glabra* inhibit methanogens growth in pure culture (McSweeney, personal communication), and the results from the Rusitec study (Chapter 4) suggest that these effects on the methanogen population persist for at least 21 days, as methanogen numbers remained low throughout when *E. glabra* was part of fermentation substrate. The extent of the reduction in methanogen population was also directly related to the substitution level of *E. glabra*. In the rumen, cellulolytic bacteria, e.g. *Ruminococcus albus*, are major hydrogen producers (Ntaikou et al. 2009). The populations of *F. succinogenes* and *R. albus* were both inhibited by *E. glabra* during the Rusitec study (Chapter 4). Inhibition of these species would potentially decrease the digestibility of the substrate and hydrogen production. The reduction in hydrogen availability results in less substrate supply to methanogens, which would indirectly influence the methanogen population and/or their activity. The population of *R. albus* recovered after two weeks of incubation of *E. glabra* in the substrate. This increase may have compensated for the loss of *F. succinogenes* and may explain why dry matter disappearance was not affected in the Rusitec study. These results suggest that *E. glabra* may impair fibre degradation initially, but all members may not be affected, plus there may be an adaptation by the ruminal microbes over time. Fermentation in the rumen is performed by a complex microbial ecosystem that contains at least 22 predominant species of ruminal bacteria (Krause & Russell 1996). The current research monitored only a few of them so it is hard to give a conclusive explanation of the interactions among the microbes and their metabolic products, particularly relating to hydrogen release and utilization. It is difficult to separate the direct and indirect effects of *E. glabra* on methanogen numbers

and activity and the degree to which each is responsible for the reduction in CH_4 production from this study. However, we have evidence from work with pure cultures of methanogens that *E. glabra* inhibits methanogens directly. A metagenomic analysis of the microbial ecology of samples taken from animals fed *E. glabra* was outside the scope of this study, but would help overcome the gap in our existing knowledge about the mechanism behind the beneficial effects that low level of *E. glabra* have on rumen fermentation. It is difficult to use this information to extrapolate to the *in vivo* study with confidence. However, the results from the *in vivo* study (Chapter 5) support the idea that the species responsible for fibre degradation in the rumen adapt to the presence of low levels of *E. glabra* in such a way that feed digestibility is not reduced.

The third significant finding from this study is that I have identified fractions from *E. glabra* that contain the antimethanogenic effects, as confirmed in batch culture tests (Chapter 6). One of the keys to understanding the mechanism of action of *E. glabra* is identifying the compound or compounds responsible for the antimethanogenic effects. It is easier to use a purified compound to investigate potential target sites for inhibition than to use the whole plant or crude plant extracts. There are additional benefits of using the knowledge about the compound(s) responsible for the bioactivity to identify other likely candidate plant species and as a molecular indicator of the antimethanogenic potential of a pasture system throughout the year and in relation to the physiological state of the plants. Currently, any measurement or assessment of variation in bioactivity in response to season, phenology, grazing is done by measuring it in the *in vitro* fermentation system. A more accurate and cost effective way to measure the variability and assess the antimethanogenic potential would be to measure the compound(s) that is directly responsible for the effects. The work undertaken in Chapter 6 represents the first stage of identifying the specific fraction(s) from *E. glabra* responsible for the antimethanogenic bioactivity. This is the first attempt to purify compounds from an Australian native plant for their use in animal production and has narrowed down the scope for defining the effective compounds and identifying them in future studies. The conclusion based on the experience from this study is that it is critical to standardize the extraction procedure for obtaining fractions or compounds that are responsible for methanogenesis.

The major challenge of this work was to determine a suitable level of *E. glabra* to feed to animals based on the *in vitro* studies (batch culture and Rusitec), which would reduce

CH_4 emissions but avoid any potential adverse side effects of *E. glabra* such as the general antimicrobial effects that may have on the animals. I found the antimethanogenic effects were consistent between the *in vitro* and *in vivo* experiments (except for the absolute value of the CH_4 reduction rate). In this thesis, I examined the persistency of the antimethanogenic effect of different levels of *E. glabra* in the Rusitec and established that an appropriate level of inclusion of *E. glabra* in a fibrous diet was 15%, before conducting the *in vivo* experiment. The control substrate used in the Rusitec study was the same as the control diet I anticipated using in the animal house experiment. The choice of 15% was based on the amount and concentration of VFA produced, as well as the other indicators of fermentation characteristics that were measured, in the treatments compared to the control substrate. These indicators were used as a guide to the degree to which the general antimicrobial effects of *E. glabra* were inhibiting overall rumen fermentation. Based on the results from this study, testing novel, bioactive plants in a Rusitec system can be valuable for designing *in vivo* studies.

As outlined earlier, there was some limitation translating the *in vitro* findings to *in vivo*. The differences between Rusitec and *in vivo* studies are well established in sheep (Mart ńez et al. 2010a) and in cattle (Hristov et al. 2012). I observed a greater concentration of propionate, butyrate and ammonia, greater DM digestibility and lower A:P in the Rusitec study compared with the *in vivo* results from the animal house experiment, which agrees with Mart ńez et al. (2010a). Contrary to Mart ńez et al. (2010a), there was higher acetate concentration in present Rusitec than *in vivo*, which is likely due to the diet difference. These differences are readily explained because the Rusitec system has a low dilution rate and there is no absorption of VFA, so VFA tend to accumulate inside the fermentation vessels, which in turn accumulates hydrogen. As with all *in vitro* systems, ruminal fermentation in the Rusitec is expected to be different to the rumen of animals. The higher dilution rate in the rumen of sheep would dilute *E. glabra* to a greater extent than in the Rusitec. These differences explain why the reduction in CH_4 production in the Rusitec is likely to have overestimated what was measured *in vivo* and why there was likely to be different impacts on the ruminal microbes and the end products of fermentation. However, the advantages of using a Rusitec experiment to test novel plants with potential bioactivity prior to testing it *in vivo* are that the procedure is more cost effective than using animals, it requires a small amount of test substrate, it is relatively easy to operate and a number of different treatments and scenarios can be tested at the same time. The results from this study

demonstrate that it can also be used to assess the more general antimicrobial effects of a novel plant species and the potential of the plant to shut down rumen function and adversely affect the animal's health and welfare. The Rusitec should be considered as a bridging step for confirming bioactivity and the persistency of effects on microbial fermentation between the crude batch culture screening of novel substrates and animal experimentation. Importantly, I have demonstrated, despite the limitations of *in vitro* methodologies for evaluating feed types, an effective process of evaluating a novel plant, rich in plant secondary compounds with the potential to inhibit CH_4, from determining a dose-response curve in batch culture, confirming the effects of the selected levels in Rusitec, and using that information to guide decisions on the level to feed animals.

In conclusion, *E. glabra* can modulate rumen fermentation and rumen microbial communities, and at the right level of intake can have a positive impact on sheep industry by reducing CH_4 without reducing productivity. This is of most interest where new grazing systems based on Australian native shrubs that are being developed to contribute to and improve year round feed supply, particularly in Mediterranean climate where there is a feed gap during summer and autumn. This study also demonstrate the value of using *in vitro* techniques to test novel feed types that are bioactive prior to testing *in vivo* and provides a process that can be followed for evaluating other natural CH_4 inhibitors. One recommendation that could be made from the study is to include a Rusitec study between a batch *in vitro* study and testing the novel substrate *in vivo*. This step is even more critical where there is scarce information about the plant or inhibitor and there is the potential to adversely affect the health and welfare of the animals if fed. The results from the microbial profiling and compound extraction work undertaken in this thesis are not yet conclusive, but they have been informative and provided a foundation for future work. A complete metagenomic analysis of the ruminal microbial ecology in animals fed 15% *E. glabra* is required to understand how *E. glabra* affects hydrogen diversion between the hydrogen users and producers, the exact nature of the antimicrobial effects of *E. glabra*. It still remains unclear exactly which phytochemicals in *E. glabra* act against methanogenesis and further work is required to isolate and identify these to help understand the mechanism of action behind the bioactivity of *E. glabra* at a microbial and cellular level. This information would be invaluable for designing a more targeted approach to the inhibition of methanogens. The purified compound or compounds may provide opportunities to develop a feed additive that reduces CH_4 emissions directly in intensive livestock systems. However, the focus of

the experiments in this study has been on improving our understanding about the potential value of *E. glabra* in grazing systems and collecting information to help guide the development of grazing management practices that ensure the full value of *E. glabra* is achieved. The results in this thesis are novel and have contributed to our understanding of how the Australian native shrub *E. glabra* affects methanogenesis and animal performance, and how rumen microbes respond to *E. glabra*. Understanding the potential value of *E. glabra* as a component of the feedbase is beneficial to researchers and farmers trying to develop novel grazing systems for Australian conditions that are both profitable and reduce enteric CH_4 emissions.

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