Research Article

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In vitro screening of selected feed additives, plant essential oils and plant extracts for rumen methane mitigation

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Abstract

BACKGROUND: Ruminants produce large quantities of methane in their rumen as a by-product of microbial digestion of feed. Antibiotics are added to ruminant feed to reduce wasteful production of methane; however, this practice has some downsides. A search for safer and natural feed additives with anti-methanogenic properties is under way. The objective of this research was to examine selected feed additives, plant essential oils and plant extracts for their anti-methanogenic potential in the rumen using an *in vitro* batch fermentation system.

RESULTS: A significant reduction ($P < 0.05$) in methane production was observed with nine feed additives (up to 40% reduction), all eight essential oils (up to 75% reduction) and two plant extracts (14% reduction) when compared to their respective controls. Amongst these, only an algal meal high in docosahexaenoic acid, preparations of *Nannochloropsis oculata*, calcareous marine algae, yeast metabolites and two tannins did not inhibit microbial gas and volatile acid production.

CONCLUSIONS: The current study identified some potent dietary ingredients or plant compounds that can assist in developing novel feed additives for methane mitigation from the rumen.

Keywords: plant extracts; essential oils; feed additives; rumen methane

INTRODUCTION

Ruminants produce large quantities of methane in their rumen as a by-product of the microbial digestion of consumed feed. Methane is a potent greenhouse gas and for this reason, much of recent research has focused on reducing ruminant methane production.[1] Feed additives are ingredients added to animal diets to improve food quality, promote growth, breakdown anti-nutritive factors, adsorb toxins, alleviate nutrient deficiencies and reduce energy-wasteful processes including production of methane in the rumen. While inclusion of antibiotic additives such as monensin to feed has been reported to reduce methane emissions *in vitro*,[2] results *in vivo* have not been consistent.[3] The use of antibiotics as feed additives is also associated with risk of developing antibiotic resistance in human pathogens,[4] and hence a search for safer components with anti-methanogenic properties is under way.[5] A variety of natural substances have been investigated as additives for livestock feeds, including yeasts, marine algae, plant derivatives and industry by-products,[6] but to date, only a few have been examined for their effect on rumen methane production.[7] Various marine products, in particular those rich in docosahexaenoic (DHA, C22:6ω-3) and eicosapentaenoic (EPA, C20:5 ω-3) acid have demonstrated significant anti-methanogenic potential.[8,9] While fish oil is abundant in these fatty acids, due to certain limitations for its use in ruminant feeds, various marine microalgae (i.e. *Crypthecodinium cohnii*, *Schizochytrium* sp., *Nannochloropsis* sp.) have been examined as potential source of these fatty acids.[10] Another interesting group of marine-based feed additives are calcareous marine algae products that are rich in calcium and magnesium and have been investigated as rumen buffers,[11] but their effects on methanogenesis have not been documented. Several yeasts have also demonstrated promising results in methane mitigation.[12] Further, industry by-products, such as various kernels and nut shells,[13] or their extracts[14] can have moderating effects on rumen methane production. Almond hulls have been examined as a potential feed for ruminants.[15] They also contain bioactive compounds such as linolenic and linoleic acid, tannins and/or triterpenoids[16] and antimicrobial

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properties are documented,[17] hence may have effect on rumen methane production. Finally, a group of additives that has received interest in recent years consists of plant-derived extracts.[18] A range of essential oils (EOs) has been reported to reduce methane production *in vitro*.[19] Some success with other plant extracts, in particular tannins,[20] saponins[21] or flavonoid-containing plant extracts[22] has also been reported. In Australia, selected species from the genus *Acacia*, *Eremophila*, *Kennedia* and *Rhagodia* have been reported as moderating methane production *in vitro*,[23] but the mechanism of this effect, i.e. if the anti-methanogenic effect related to specific bioactivity of extractable compounds or simply due to overall poor fermentability has not yet been examined.

The aim of this study was to test selected feed additives, as well as EOs and ethanolic extracts derived from Australian plants for their potential to reduce methane production by sheep rumen microbes *in vitro*.

MATERIALS AND METHODS

Fermentation substrates and additives

The testing incorporated a range of feed additives ($n = 11$), plant EO ($n = 8$) and ethanolic extracts ($n = 10$), included at concentrations per manufacturer's specification (for feed additives), or at concentrations previously reported in the literature (for plant EOs and ethanolic extracts) to be effective at suppressing rumen microbes.[24] The treatments and inclusion concentrations are listed in Table 1. As these *in vitro* studies aim to be relevant to intensive farming systems where animals are fed grain-based diets, we opted to use this type of diet as fermentation substrate. The control fermentation substrate was therefore a commercial pellet (Milne Standard Pellets, Milne Feed, Welshpool, Western Australia, Australia) which contained barley (350 g kg^{-1}), oats (200 g kg^{-1}), wheat (200 g kg^{-1}), lupins (60 g kg^{-1}), straw (100 g kg^{-1}), mill mix (50 g kg^{-1}) and minerals (40 g kg^{-1}); and had nutritive value of dry matter (DM) 910 g kg^{-1} DM, acid detergent fibre 156 g kg^{-1} DM, neutral detergent fibre 282 g kg^{-1} DM, crude protein 145 g kg^{-1} DM and fat 12 g kg^{-1} DM. Pellet was ground to pass a 1 mm screen prior to inclusion in the assay. Commercial feed additives were obtained as follows: monensin (Rumensin 100 Elanco) from Advanced Feeds, Midvale, Australia; marine-based product 1 (commercial product DHA-Gold, a dried algal meal containing 460 g kg^{-1} DHA) from Martek Biosciences Corp, Columbia, MD, USA; marine-based product 2 (extract from *Nannochloropsis oculata* NQAIF283, isolated by the North Queensland Algal Identification/Culturing Facility, curator Stan Hudson, harvested in stationary phase and lyophilised, containing 215 g kg^{-1} EPA) from MBD Energy and the Advanced Manufacturing CRC, Melbourne, Victoria, Australia; Tannin 1, 2, and 3 from Mimosa Central Cooperative Ltd, Pietermaritzburg, South Africa; almond hulls from Castlegate James Australasia Pty Ltd, Robinson, Victoria, Australia; commercial EO blend 1 (Agolin® Ruminant), EO blend 2 (CRINA Ruminants), yeast metabolites (Diamond V Diamune) and marine-based product 3 (Acid Buff, calcareous marine algae) from Feedworks Pty Ltd Romsey, Victoria, Australia; and eight plant EOs from The Paperbark Co., Harvey, Western Australia, Australia. Furthermore, crude ethanolic plant extracts were obtained 'in-house' from 10 Australian native plants grown at an experimental site in South Australia. The plant traits, collection sites, plant stage of growth at collection time and post-harvest processing have been described previously[23] and extracts were obtained by a procedure based on that described by Hutton *et al.*[24] Briefly, 5 g of plant material were placed in a centrifuge tube and mixed with 20 mL of 70/30 ethanol–water (v/v) and the mixture was macerated for 3 h with shaking at 100 cycles per min, at 22°C. This mix was then centrifuged at 4946 × *g* for10 min and the supernatant was transferred to a separate tube. Four millilitres of 70/30 ethanol–water (v/v) was added to macerated residue and macerated again for 1 h with shaking at 12 × *g* at 22°C and the supernatants were combined. Ethanol was evaporated at 40–50°C using a vacuum evaporator (Rotavapor-R; Buchi, Flawil, Switzerland). Prior to testing, extracts were reconstituted in 70/30 ethanol–water (v/v) to a concentration of 100 g L^{-1}. The control consisted of fermentation substrate + 100 μL 70/30 ethanol-water (v/v).

In vitro fermentation technique

All experimental material was tested using an *in vitro* batch fermentation assay.[23] Each treatment was tested in triplicate. Briefly, 0.1 g of fermentation substrate (control) was weighed in triplicate into specialised anaerobic culturing tubes (Anaerobic Tube, cat. No 2048-00150; BellCo Glass, Vineland, NJ, USA) and transferred into an anaerobic chamber (Coy Vinyl Anaerobic Chamber; Coy Laboratory Products Inc., Grace Lake, MI, USA, maintained at 39°C and supplied with 800 mL L^{-1} N_2, 100 mL L^{-1} CO_2 and 100 mL L^{-1} H_2). The rumen fluid was collected on the day of the experiment from two rumen-fistulated sheep, 2 h after feeding to obtain a sample with a maximal microbial activity. Rumen fluid was pooled, strained and buffered to pH 7.1–7.3 using McDougall buffer and each tube was filled with 10 mL of this buffered rumen fluid. Additives (except for almond hulls) were separately dissolved in 1 mL buffered rumen fluid to make a stock solution, and then 100 μL of this mixture was added to the treatment tube containing substrate and buffered rumen fluid (Table 1). For almond hull treatment, 0.05 g of control substrate was mixed with 0.05 g almond hulls. Each run also included batch controls – buffered rumen fluid and a positive control (oaten chaff + buffered rumen fluid) – that allowed the detection of any abnormalities with donor rumen fluid. Tubes were stoppered, crimped and incubated with shaking for 24 h. At the end of incubation period, gas pressure and methane concentrations in the headspace gas, as well as concentrations of volatile fatty acids (VFA), ammonia and acetate: propionate ratios were measured as described previously.[23] Methane concentrations were calculated and expressed as mL g^{-1} DM incubated (DMi) and as mol mol^{-1} of VFA.

Statistical analysis

All data were analysed using JMP® software and the treatment responses in gas production, VFA, CH_4 and ammonia concentrations were examined in separate models with treatment as a factor. Each factor had three observations (i.e. separate vials) and data were analysed performing one-way analysis of variance and treatment as a fixed effect: $Y_{ij} = \mu + T_i + E_{ij}$, where Y_{ij} was the observation, μ was the overall mean for each parameter, T_i was the effect of treatment and E_{ij} was residual error. Least significant difference (LSD) was used to compare the treatments to respective controls and significant differences were declared at $P < 0.05$.

RESULTS

Feed additives

Inclusion of nine feed additives with control resulted in significant decreases ($P < 0.05$) in methane production, with the level of reduction between 30% and 40% (26–38 mL methane g^{-1} DMi)

Table 1. Fermentation products resulting from substrate incubated when incubated *in vitro* with buffered sheep rumen fluid in the presence of different additives

Treatment	Amount per tube	Fermentation product (mmol L^{-1})					
		Gas (kPa)	VFA (mmol L^{-1})	CH_4 (mL g^{-1} DMi)	CH_4 (mol/mol VFA)	A:P	NH_3 (g L^{-1})
Control substrate[1]	100 mg	103	86	46	0.24	2.3	0.29
Control substrate + 100 µL EtOH[2]	100 mg	104	101	44	0.19	2.7	0.27
Feed additives							
Monensin	0.05 mg	103	86	44	0.23	2.2	0.31[a]
Marine algae product 1	16.4 mg	99	89	33[b]	0.17[b]	2.2	0.33[a]
Marine algae product 2	16.4 mg	95	81	27[b]	0.15[b]	2.2	0.38[a]
Marine algae product 3	0.8 mg	95	86	33[b]	0.17[b]	2.3	0.31[a]
Yeast metabolites	0.22 mg	96	88	35[b]	0.18[b]	2.4	0.31[a]
Almond hulls[3]	50 mg	90[b]	91	34[b]	0.17[b]	2.5[a]	0.20[b]
Tannin 1	20 mg	86[b]	86	26[b]	0.13[b]	2.4[a]	0.27
Tannin 2	20 mg	91	90	38[b]	0.19[b]	2.5	0.39[a]
Tannin 3	20 mg	90[b]	89	37[b]	0.19[b]	2.6[a]	0.37[a]
Commercial EO blend 1	0.01 mg	91	85	32[b]	0.17[b]	2.4	0.31[a]
Commercial EO blend 2	0.01 mg	106	79	46	0.26	2.2	0.31[a]
Essential oils							
Agonis fragrans	25 µL	69[b]	54[b]	26[b]	0.21	4.6[a]	0.26[b]
Eucalyptus plenissima	25 µL	73[b]	56[b]	30[b]	0.24	5.6[a]	0.24[b]
Eucalyptus staigeriana	25 µL	54[b]	41[b]	13[b]	0.14[b]	3.9[a]	0.22[b]
Leptospermum pettersoni	25 µL	53[b]	44[b]	14[b]	0.14[b]	4.3[a]	0.20[b]
Melaleuca alternifolia	25 µL	78[b]	62[b]	31[b]	0.22	5.7[a]	0.25[b]
Melaleuca ericifolia	25 µL	58[b]	51[b]	11[b]	0.10[b]	4.1[a]	0.25[b]
Melaleuca teretifolia	25 µL	37[b]	37[b]	11[b]	0.13[b]	3.9[a]	0.22[b]
Santalum spicatum	25 µL	74[b]	80[b]	25[b]	0.14[b]	2.0[b]	0.28
Extracts from Australian plants							
Acacia saligna	100 µL	85[b]	92	50	0.24[a]	2.5[b]	0.14[b]
Atriplex nummularia	100 µL	107	109	54[a]	0.22	2.9	0.27
Chameacytisis palmensis	100 µL	111	114[a]	50	0.20	3.0[a]	0.25[b]
Cullen australasicum	100 µL	95	98	48	0.22	2.5[b]	0.27
Enchylaena tomentosa	100 µL	102	99	57[a]	0.26[a]	2.7	0.26
Eremophila glabra	100 µL	91[b]	98	38[b]	0.17[b]	2.2[b]	0.23[b]
Eremophila longifolia	100 µL	115[a]	102	47	0.21	2.7	0.19[b]
Kennedia prorepens	100 µL	96	106	38[b]	0.16[b]	2.7	0.15[b]
Maireana brevifolia	100 µL	106	107	53[a]	0.22	2.9[a]	0.30[a]
Rhagodia preissii	100 µL	104	92	54[a]	0.26[a]	2.7	0.22[b]
SEM		3.7	3.3	2.4	0.030	0.2	0.02

[1]Control for feed additives and essential oils.
[2]Control for plant extracts.
[3]Almond hulls treatment: 50 mg almond hulls and 50 mg control substrate.
A:P, acetate: propionate.
[a,b]Within the same column and in same experiment [a]significantly higher than the respective control [b]significantly lower than the respective control ($P < 0.05$). VFA, volatile fatty acids.

when compared to the control without the additive (46 mL methane g^{-1} DMi, Table 1). Amongst these, only three additives also inhibited gas production, but VFA concentrations were unaltered in all treatments. There was no strong correlation between VFA and methane ($R^2 = 0.01$) and nine additives produced less methane per mol of VFA compared to control ($P < 0.05$). The acetate: propionate ratio and ammonia concentrations were lowered only with the addition of Tannin 1 and almond hulls.

Essential oils

All eight essential oils caused significant reductions ($P < 0.05$) in methane concentrations, with methane yields ranging from 11 mL g^{-1} DMi to 31 mL g^{-1} DMi, compared to control (44 mL g^{-1} DMi). The EOs from both *Melaleuca ericifolia* and *M. teretifolia* substantially (i.e. 75%) inhibited methane production. All of the EOs significantly reduced gas and VFA production, with some correlation between VFA and methane ($R^2 = 0.47$), but there were five EOs that produced significantly less methane per mol of VFA compared to control ($P < 0.05$). All of the EOs, except *Santalum spicatum*, increased acetate: propionate and reduced ammonia levels.

Plant extracts

The addition of plant extracts from *Eremophila glabra* and *Kennedia prorepens* resulted in significant ($P < 0.05$) decreases in methane

yield (38 mL g^{-1} DMi each) compared to the control (44 mL g^{-1} DMi, Table 1). These two plant extracts also caused significant ($P < 0.05$) reductions in production of gas, but not in VFA concentrations and produced less methane per mol of VFA compared to control ($P < 0.05$) In general, there was no correlation between VFA and methane ($R^2 = 0.001$). Extracts from *Acacia saligna*, *E. glabra* and *Cullen australasicum* also significantly reduced acetate: propionate, while extracts from six plant species also reduced ammonia production.

DISCUSSION

Recent reviews have identified a significant potential of natural plant extracts as a source of novel, antibiotic-free feed additives for methane mitigation in ruminants.[5,8,12] The current study examined a range of products that can potentially be added to a concentrate-based ruminant diet, with the aim of reducing some wasteful microbial processes in the rumen. This research has shown that nine commercial feed additives, eight plant EOs and two plant extracts, when combined with a grain-based diet and incubated *in vitro*, resulted in significant reductions in methane production by rumen microbes. Amongst these, six also did not inhibit overall rumen fermentation and 16 produced less methane per unit of VFA, indicative of more specific effect on methanogenesis. Amongst feed additives, the most potent anti-methanogenic one was Tannin 1 from *Acacia mearnsii*, which caused 43% reduction in methane production, accompanied by 17% reduction in total gas production. This reduction can be almost entirely accounted for by the reduction in methane. The magnitude of this reduction in methane production in response to tannin in *Acacia* is similar to that reported by others *in vitro*[25] and *in vivo* in sheep[26] and cattle.[20] Thus, the finding not only aligns with previous research, but supports the contention that our *in vitro* technique mimics methanogenesis *in vivo*. However, there is some evidence that the effects of tannins may be short-lived[27] and further studies in a continuous culture system are needed to examine any adaptation of methanogen microbes and rule out any detrimental effect of this additive on rumen fermentation.

Another set of additives that substantially reduced methane production were marine algae-based products. The commercial product 1 used in this investigation was a marine algae containing a high concentration of DHA. Our results with this product are consistent with the *in vitro* based findings of Fievez *et al.*,[9] who reported that methane production was suppressed by up to 80% by inclusion of a similar DHA-containing product and attributed the effect to the high concentration of DHA in this product (i.e. 200 g DHA kg^{-1} DM). However, our results are at variance from recently published research that showed there was no effect on methane emissions when this same algal meal was fed to dairy cows.[28] The other marine-based product tested, i.e. marine algae product 2, obtained from *N. oculata*, which contained a high concentration of EPA (215 g kg^{-1} total fat) and some DHA (32 g kg^{-1} fat), caused even greater decrease in methane production. This aligns with findings of Fievez *et al.*,[8] who reported considerable reduction in methane production *in vitro* using EPA and DHA-rich fish oil as an additive. Thus, the evidence suggests that these two fatty acids that are present in marine algae are potent anti-methanogens *in vitro*, but further studies are required to confirm their effect *in vivo*.

In this study, we have identified for the first time, the ability for almond hulls to substantially inhibit methane production *in vitro* without compromising concentrations of VFA. This product is rich in bioactive compounds such as phenolics and triterpenoids,[16] which have potent antioxidant activities.[29] Some antibacterial effects of almond oil (derived from almond hulls) have been reported,[17] but the effect of almond products on rumen microbes yet needs to be examined. Further *in vitro* research is warranted to investigate the agents in almond hulls that may be responsible for inhibition of methanogenesis, while additional research is also warranted to determine if this effect also occurs *in vivo*.

The ionophore monensin, at the concentrations used in this experiment, was ineffective at reducing methane when combined with a concentrate-based diet. This finding is perhaps not surprising, since studies have reported variable success in inhibiting methane emissions from roughage-based diets,[30] while the anti-methanogenic effect with concentrate-based diets appears to be limited.[31]

A commercial blend of EO (EO 2) containing thymol, eugenol, vanillin and limonene, at doses applied here, was also ineffective at inhibiting methane production, despite the fact that individual components, i.e. thymol, have demonstrated some potential to inhibit methanogenesis.[32] However, the other commercial EO blend product (EO 1) containing eugenol, geranyl acetate and coriander oil as major components had a profound effect on methane in this study. Coriander EO has recently been reported as having anti-methanogenic properties in the rumen,[33] while geranyl acetate belongs to a class of oxygenated monoterpenes that also have potent bioactive effects,[19] and it is possible that the effect may be associated with these two compounds.

Essential oils from Australian plants were also highly effective in reducing methane production, with reductions up to 75% from EOs extracted from *Melaluca teretifolia* and *Melaleuca ericifolia*. *M. teretifolia* contains the highest neral and geranial concentrations of all Melaleucas, while a major secondary constituent in *M. ericifolia* is linalool, all known to have antimicrobial properties.[34] It has also been reported that terpenoids remain undegraded in the rumen for a prolonged period,[35] hence their activity is extended. However, all EO tested here also inhibited overall microbial activity, a similar finding having previously been reported,[36] suggesting that inclusion of EOs in diets at these doses could compromise energy supply to ruminants. In contrast, some studies have reported that certain EOs, at carefully selected doses, showed no effect on VFA[37] or may even increase VFA production,[38] implying dependence of the outcome on the type of the EO as well as other conditions such as the dose and the diet.[31] Ideally, the additives should reduce methane production, without interfering in the overall fermentation processes, so further studies on dose responses to these EOs are warranted.

Plant extracts, even when selected based on their known bioactivity, often have limited effect on rumen methanogenesis. For example, amongst 450 extracts tested, Bodas *et al.*[5] found only 15 anti-methanogenic plant extracts, and amongst these, only six that had no effect on overall fermentation. In our study, out of 10 ethanolic plant extracts, only two, i.e. *E. glabra* and *K. prorepens*, were capable of reducing methane production from *in vitro* fermentation. These two plants have previously been identified as the most potent anti-methanogenic plants amongst 120 Australian native shrubs tested.[23] In that study, when these two plants were incubated *in vitro* as sole substrates in a dried/ground form, methane production was significantly reduced, but so was total gas production. In contrast, in the current study, when ethanolic extracts of these two plants were included in incubations, methane production was significantly reduced, but total gas production was not. We may speculate regarding the causes for these observations, but the principal conclusion is that the methane inhibitory agent(s)

in *E. glabra* and *K. prorepens* are ethanol-soluble and that these two plants probably contain ethanol insoluble compounds that inhibit overall rumen fermentation. Recently, we have isolated some potent bioactive compounds from *E. glabra* that inhibit specific rumen bacteria, i.e. *Streptococcus bovis*.[24] Re-isolating and testing of compounds for anti-methanogenic properties is under way and further research will reveal if similar effects exist against rumen methanogens, or if the reduced methanogenesis is result of hydrogen being utilised by other microbial pathways. For example, it is often implied that propionate production increases when methane production is inhibited.[39] However, in our study, only three treatments had this effect, with trends observed with *E. glabra* extract aligning with our previous observations when *E. glabra* plant was used.[23] In the current study, reduced methane was coupled with increased ammonia concentrations in 10 treatments only, implying that some alternative hydrogen sinks were operating.

We would like to point out that the amounts of EOs or extracts applied here correspond to a relatively high amounts/concentrations of plant material *in situ*, these amounts/concentrations having been chosen to be consistent with those used by other researchers.[19,24] However, many factors may affect bioactivity of additives *in vitro* and may not necessarily directly translate to amounts/concentrations that would be applicable *in vivo*.[40] Further, we have identified some additives that have potent anti-methanogenic properties when tested *in vitro* with a concentrate-based diet, but their effects when mixed with forage-based diets are yet to be examined. Therefore, results reported here currently may only apply to conditions used, i.e. *in vitro*, using rumen fluid from merino sheep and with one type of concentrate diet and further studies are needed to confirm these findings over a range of conditions and *in vivo*.

CONCLUSIONS

This study identified a number of feed additives and products that, when combined with a concentrate-based diet, reduced methane production from the rumen microbes *in vitro*, with six additives not impeding with overall fermentation. The variation in responses between products tested here provides the potential to select and develop some new anti-methanogenic additives. Further investigations are required to quantify the persistency of these effects over time and under a variety of conditions. It is also necessary to confirm the enteric anti-methanogenic potential of these feed additives *in vivo*, as well as their suitability as feed supplements for ruminants.

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