The red macroalgae Asparagopsis taxiformis is a potent natural antimethanogenic that reduces methane production during in vitro fermentation with rumen fluid
The red macroalgae *Asparagopsis taxiformis* is a potent natural antimethanogenic that reduces methane production during *in vitro* fermentation with rumen fluid

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Abstract. Livestock feed modification is a viable method for reducing methane emissions from ruminant livestock. Ruminant enteric methane is responsible approximately to 10% of greenhouse gas emissions in Australia. Some species of macroalgae have antimethanogenic activity on *in vitro* fermentation. This study used *in vitro* fermentation with rumen inoculum to characterise increasing inclusion rates of the red macroalga *Asparagopsis taxiformis* on enteric methane production and digestive efficiency throughout 72-h fermentations. At dose levels ≤1% of substrate organic matter there was minimal effect on gas and methane production. However, inclusion ≥2% reduced gas and eliminated methane production in the fermentations indicating a minimum inhibitory dose level. There was no negative impact on substrate digestibility for macroalgae inclusion ≤5%, however, a significant reduction was observed with 10% inclusion. Total volatile fatty acids were not significantly affected with 2% inclusion and the acetate levels were reduced in favour of increased propionate and, to a lesser extent, butyrate which increased linearly with increasing dose levels. A barrier to commercialisation of *Asparagopsis* is the mass production of this specific macroalgal biomass at a scale to provide supplementation to livestock. Another area requiring characterisation is the most appropriate method for processing (dehydration) and feeding to livestock in systems with variable feed quality and content. The *in vitro* assessment method used here clearly demonstrated that *Asparagopsis* can inhibit methanogenesis at very low inclusion levels whereas the effect *in vivo* has yet to be confirmed.

Additional keyword: greenhouse gas, ruminant, seaweed.

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Introduction

Methane (CH\(_4\)) in the atmosphere is a potent greenhouse gas (GHG) with an IPCC Fifth Assessment Report (AR5) global warming potential 28 times that of carbon dioxide (CO\(_2\), IPCC 2014). Between 2000 and 2009, agriculture and waste management accounted for 62% of global anthropogenic CH\(_4\) emissions (Kirschke *et al*. 2013) with ruminant enteric fermentation responsible for 58% of agricultural contributions (Olivier *et al*. 2005). In Australia, the contribution of CH\(_4\) from ruminant livestock is approaching 10% of total GHG emissions (Henry *et al*. 2012). These levels have resulted in a universal effort to reduce enteric CH\(_4\) emissions. Enteric CH\(_4\) is a consequence of anaerobic fermentation of feed organic matter (OM) by a microbial consortium that produces substrate CO\(_2\) and hydrogen in a reduction pathway used by methanogens (Morgavi *et al*. 2010). Feed additives have been used to interfere with this pathway or otherwise reduce the numbers of functional methanogens. Patra (2012) reviewed dietary supplementation options for rumen enteric CH\(_4\) management that included ionophores, chemical compounds, legumes, essential oils, fats, saponins, tannins, probiotics, and plant secondary metabolites. Unfortunately, an antimethanogenic effect may be concomitant with some detrimental impacts. Most commonly there is a decrease in fermentation efficiency leading to a decrease in feed intake and a measurable decline in animal productivity.

Macroalgae also have potential for use as a supplement for livestock feeds (Machado *et al*. 2015). The antimethanogenic properties of macroalgae-based functional products in rumen *in vitro* cultures has been demonstrated (Wang *et al*. 2008; Dubois *et al*. 2013; Kinley and Fredeen 2015; Machado *et al*. 2014), however there is much variability in the antimethanogenic potency between types and species of macroalgae. Algae are generally classified by size (micro or macro) whereas macroalgae are broadly classified based on pigmentation (green, red or brown) and habitat (freshwater or
Asparagopsis eliminates methane from fermentations

One novel antimethanogenic strategy seeks to harness the effect of secondary metabolites found in some macroalgae. These have been demonstrated to be variable in effect on in vitro fermentation in a dose-dependent manner (Dubois et al. 2013; Machado et al. 2015a). Machado et al. (2014) reported the antimethanogenic effect of 20 different macroalgal species in vitro when fermented with a low quality dry rangeland Rhodes grass (Chloris gayana). This work demonstrated that at high inclusion rates (17% OM basis) highly variable effects on methanogenesis were possible. Asparagopsis is a marine genus of red macroalgae characterised by secondary metabolites with antibacterial properties (Paul et al. 2006) that demonstrates a potent antimethanogenic effect in vitro (Machado et al. 2015b).

It was hypothesised that the red macroalga Asparagopsis taxiformis at low inclusion rates can dramatically reduce CH4 emissions from in vitro fermentations with rumen fluid (RF) without detrimental effects on fermentation while using a grass feed substrate. The objective of this study was to demonstrate the in vitro antimethanogenic potency of the red macroalga Asparagopsis taxiformis at low inclusion rates over 72 h using an irrigated Rhodes grass as the feed substrate. The effects on parameters of rumen fermentation were examined using standardised in vitro culture methods.

**Materials and methods**

**Preparation of macroalgae and Rhodes grass substrate**

The Asparagopsis taxiformis (hereafter Asparagopsis) was harvested from Nelly Bay, Magnetic Island (19°16′S, 146°58′E) near Townsville, Qld, Australia. The macroalgal biomass was rinsed in seawater for 2 min then dipped in freshwater to remove residual salt to maximise alga OM content. The clean biomass was placed in 100-μm mesh and centrifuged at 1000g for 6 min at ambient temperature in a commercial washing machine to remove excess water and then stored at −10°C. The biomass was then freeze-dried (SP Industries VirTis K, Warminster, PA, USA) and ground to 1 mm and stored at −10°C. The high quality Rhodes grass (HQR) was grown under irrigation and harvested locally. Subsamples of HQR were air-dried and ground to 1 mm. Table 1 describes the composition of the Asparagopsis and HQR biomass used as the fermentation substrates. Dry matter was determined by achievement of constant weight at 105°C, and OM was measured as loss on combustion at 550°C for 8 h (Horwitz 2000). Neutral and acid detergent fibre were determined using an Ankom (Macedon, NY, USA) model 200 fibre analyser. Crude protein content was determined using a LECO (St Joseph, MI, USA) model CHN628 series nitrogen analyser.

| Table 1. Nutritional composition of the Rhodes grass substrate and Asparagopsis biomass (g/kg DM unless stated otherwise) |
|----------------------------------|------------------|------------------|
| Composition                      | Rhodes grass     | Asparagopsis     |
| Dry matter (g/kg as used)        | 916              | 945              |
| Organic matter                   | 878              | 811              |
| Crude protein                    | 167              | 252              |
| Neutral detergent fibre*         | 645              | –                |
| Acid detergent fibre             | 315              | –                |

*Without α amylase.

**Donor animals and preparation of RF inoculum**

Rumen fluid inoculum was collected from four fistulated Brahman steers (Bos indicus; LW 490 ± 45 kg) fitted with 10-cm Bar Diamond (Parma, OH, USA) rumen cannulas. The steers were maintained at the College of Public Health, Medical and Veterinary Sciences at James Cook University (Townsville) according to current guidelines (NHMRC 2013) and approved by the local animal ethics committee (A5/2011). The steers were maintained on Rhodes grass ad libitum for 6 months before the collection of RF, which was extracted 2 h after morning feeding by sampling from four quadrants of the rumen and hand-squeezing to completely fill pre-warmed 1-L stainless steel thermal flasks.

**Inoculation and in vitro fermentation**

The RF was pooled and immediately processed by filtration through a 0.5-mm sieve and combined with Goering and van Soest (1970) buffer (GVB) at a ratio of 1:4 (RF:GVB). Maintenance of 39°C and mixing of the RF buffer fermentation media (RFB) was continuous to ensure homogeneity (Major Science SWB 20 L-3; Saratoga, CA, USA). The full system was N2 purged and a Dose-It pump (Integra Biosciences, Hudson, NH, USA) was used to aspirate 125 mL of RFB into incubation bottles containing the Asparagopsis and HQR. The bottles were sealed with an Ankom RF1 gas production module (Macedon, NY, USA) and placed in an incubator (Ratek OM11; Boronia, Vic., Australia) maintained at 39°C and oscillating at 85 RPM.

**Experimental design**

To characterise the effect of Asparagopsis on in vitro rumen fermentation a series of 20 incubation periods were conducted using five Asparagopsis dose rates ranging from 0.5% to 10% (OM basis) of the HQR substrate OM and compared with a control (no macroalgae) and RFB blanks. Each dose level was characterised in duplicated incubation periods containing quadruplicate repetitions at each sampling time point (12, 24, 36, 48, and 72 h). Controls and blanks were included in all periods and time points in duplicate. Each 72-h fermentation series was split into two periods with the first monitoring at 12, 24 and 36 h (plus a 6-h sample for in vitro apparent digestibility of substrate OM (IVD-OM only)) and the second monitoring 36, 48, and 72 h. The data was then combined to provide time series curves covering the full 72 h. Each fermentation contained 1.0 g of the HQR (OM basis) and appropriate quantities of
*Asparagopsis* to achieve dose rates of 0.5%, 1%, 2%, 5% and 10% according to the biomass composition described in Table 1.

**Fermentation monitoring and sample analyses**

**Total gas production**

The methods used in this study were similar to those described by Cone et al. (1996), Pellikaan et al. (2011) and Machado et al. (2014). Total gas production (TGP) was measured continuously for a maximum of 72 h. The Ankom parameter settings were kept constant with maximum pressure in the fermentation bottle of 3 psi, which when exceeded, would vent for 250 ms and the pressure change accounted in the cumulative pressure recording. Gas pressure was measured every 60 s and cumulative pressure was recorded at 20-min intervals. The cumulative TGP expressed in mL/g of substrate OM was determined by application of the natural gas law to the accumulation of the recorded gas pressure while accounting for individual bottle volume.

**Methane production**

*In vitro* CH₄ production was determined and time series production curves prepared by collection of samples at multiple time points. Production in mL CH₄/g of substrate OM was estimated by application of CH₄ concentration in time series samples using the relative TGP while assuming constant homogeneity of bottle headspace. Concentration of CH₄ in samples was measured by gas chromatography (GC) on a Shimadzu GC-2014 (Kyoto, Japan) equipped with a Restek Stabilwax (25 mL/min and injection volume was 250 μL). Methane production had a similar trend as TGP. There was no greater CH₄ production than the treatments (fixed factor) using the mgcv package within the R language (version 3.0.1; R Core Team 2013).

**Results**

*Asparagopsis* had consistent effects on *in vitro* fermentations and these effects were dose dependent. This study applied a HQR (Table 1) as substrate and the dose rate of 2% *Asparagopsis* (OM basis) was near the optimum dose as measured by decrease in TGP, CH₄ abatement, stability of IVD-OM, and beneficial changes in VFA concentrations. Using HQR, the 1% dose was no more effective than 0.5% (Fig. 1). However, there was a significant reduction in TGP of ~30% with the inclusion at 2% of *Asparagopsis* (P < 0.001). There was good reproducibility of TGP lending to standard error (s.e.) of <1.5 mL/g representing 0.7% of the TGP values at 214 mL/g substrate OM.

The inclusion of *Asparagopsis* had the effect of reducing CH₄ production in a dose and time-dependent manner (Fig. 2). Methane production had a similar trend as TGP. There was minimal CH₄ produced with 1% *Asparagopsis* inclusion in the first 24 h, however after 24 h CH₄ production began to increase rapidly. After 48 h there was no longer measurable difference in CH₄ between the 0.5% and 1% dose rates. The prominent effect occurred at dose levels ≥2% (P < 0.001) and no detectable CH₄ was produced. Variability in CH₄ production between periods was greater than that observed for other variables monitored in this study.

Substrate degradability in *in vitro* was not affected by the inclusion of *Asparagopsis* ≤5.0% (OM basis). There was no difference in IVD-OM over 72-h fermentations between the control and dose rates up to 5% of substrate OM. However, 10% *Asparagopsis* induced significant reduction in IVD-OM (P < 0.001). The comparison of IVD-OM between dose rates and the control was consistent and independent of time. The IVD-OM variability within- and between periods was small as reflected by the small s.e. (Fig. 3).

In Fig. 4 it was shown that *Asparagopsis* at doses ≤2% had little effect on total VFA (TVFA) after 72 h of fermentation and little change was induced by dose rates ≤5%, however with 5% the TVFA was decreased (P < 0.05) and more
so at 10% \((P < 0.001)\). Although TVFA was not acutely sensitive to low level inclusion of *Asparagopsis* there was a trend towards reduced TVFA with increased dose. For individual VFA a significant change was demonstrated \((P < 0.05)\) and the effect was magnified with increasing dose \((P < 0.001)\). As the dose rate of *Asparagopsis* increased through the 0.5%, 1%, 2%, 5%, and 10% dose range the acetate concentrations decreased by 4%, 11%, 29%, 41%, and 61%, respectively, compared with the control. For propionate the concentrations increased by 13%, 0%, 56%, 88%, and 106% for the same dose range, respectively. For butyrate the increases were 67% and 116% for the 2% and 10% doses, respectively. These changes in acetate and propionate concentrations reduced the acetate : propionate ratio. With little effect on TVFA and a shift to lower acetate and greater

![Fig. 1](image1.png)

**Fig. 1.** The time series effect of increasing dose rate of *Asparagopsis* on *in vitro* total gas production (mL/g organic matter). Control was a high quality Rhodes grass hay. Error bars are not shown as they were smaller in size than the symbols used.

![Fig. 2](image2.png)

**Fig. 2.** The time series effect of increasing dose rate of *Asparagopsis* on mean \(\pm\) s.e.m. *in vitro* methane production (mL/g organic matter). Control was a high quality Rhodes grass hay.
propionate, there was no negative impact on VFA production due to low dose (<5%) Asparagopsis inclusion in vitro using HQR substrate.

**Discussion**

The red seaweed *Asparagopsis* has a large CH₄ abatement capacity compared with other natural products when included at low dose in rumen fermentations in vitro. The effect of *Asparagopsis* demonstrated in this study agreed with Machado et al. (2015b), which described end-point results after 72 h of fermentation that demonstrated dose sensitivity to *Asparagopsis* using low quality Rhodes grass (LQR) substrate. Conspicuously however, in their study there was an abrupt reduction in TGP and CH₄ production occurring at 1% *Asparagopsis* rather than the 2% in this study (Figs 1, 2). Consequently, this effective dose

![Graph 3](image3.png)

**Fig. 3.** The time series effect of increasing dose rate of *Asparagopsis* on mean (+s.e.m.) *in vitro* apparent organic matter digestibility. Control was a high quality Rhodes grass hay.

![Graph 4](image4.png)

**Fig. 4.** The effect of increasing dose rate of *Asparagopsis* on mean (+s.e.m.) *in vitro* total volatile fatty acid, acetic, propionic and butyric acid concentrations after 72 h of fermentation. Control was a high quality Rhodes grass.
difference observed with variable grass quality may follow a different pattern between grass types or when grain-based substrates are used. Therefore, it is essential to evaluate the effects using the various ruminant feeding systems. Using HQR the 1% dose of Asparagopsis was no more effective than 0.5%, and thereafter TGP and CH₄ declined with increasing dose levels. It is an important distinction that the fermentation response to Asparagopsis may also be dependent upon the quality of the substrate. Therefore, the requirements for Asparagopsis biomass may be only half when feeding LQR compared with HQR. Ruminant production systems utilising low quality forage as the primary feed would require less Asparagopsis to achieve equivalent CH₄ abatement.

The production of CH₄ was virtually undetectable at dose rates ≥2% OM basis (Fig. 2). For this reason the CH₄ results were not blank corrected, doing so would produce confusing negative values because Asparagopsis-treated fermentations produced less CH₄ than the blanks. However, occasionally during other in-house rumen in vitro experiments using a 2% dose (data not shown) a small rise in CH₄ was observed after ~36 h. This occasional rise in CH₄ still provided at minimum an abatement of >85% compared with the control. Those fermentations used the HQR substrate with the only difference being the RFB. At the 1% Asparagopsis dose rate there was a typical rise in CH₄ from undetectable to ~20 mL/g HQR beginning between 24 and 36 h of fermentation. All experimental periods demonstrated that at an Asparagopsis dose rate ≥2% of substrate OM typically results in undetectable in vitro methanogenesis. At ≥5% the CH₄ production was always undetectable. Feed energy is typically lost as CH₄ at a rate of up to 12% of gross energy intake (Johnson and Johnson 1995). Using Asparagopsis this energy may be conserved in the rumen for productive use by the ruminant animal at some undefined level which further reduces the cost of abatement. This proportion of retained energy can be quantified with in vivo feeding studies that closely monitor feed intake, CH₄ production, and productivity.

It is known that the antibacterial defence mechanism of Asparagopsis is predominantly a result of the secondary metabolite bromoform (CHBr₃) naturally present in the macroalgal biomass (Paul et al. 2006). Bromoform is similar chemically and in antimethanogenic potency to that of bromochloromethane (BCM; CH₂BrCl). In previous in vivo experiments investigating enteric CH₄ abatement, BCM induced abatement in Brahman steers of 93% and 50% after separate 28 and 90 days feeding regimes, respectively (Tomkins et al. 2009). However, BCM has been banned from manufacture and use in Australia due to its contribution to ozone depletion. The mode of action of BCM was described previously as inhibition of the methyl-dependent methyl transferase step in release of CH₄ (Denman et al. 2007). In that study inhibition of methanogenesis occurred immediately however the methanogen populations were only found to be reduced after several hours, thus the observed lag in the population decline suggested that the inhibition of methanogenesis directly affected growth of methanogens. They also commented that BCM would be removed from the rumen due to ruminal flow and unless it was replaced CH₄ inhibition would decline, which could not be observed during our in vitro batch culture. However, a decline in inhibition was observed with the 1% inclusion (Fig. 2) and was presumably associated with consumption of the antimethanogenic capacity of Asparagopsis at very low dose levels.

The naturally occurring secondary metabolites in Asparagopsis armata a temperate species closely related to the tropical Asparagopsis used in the present study include di-BCM (CHBr₂Cl) at low levels of <0.1% of algal DM, but also bromoform, which at higher levels of ~1.7% (Paul et al. 2006) is considered to be the bioactive agent responsible for most of the CH₄ abatement acting in the same way as BCM. The naturally occurring secondary metabolites of Asparagopsis have demonstrated activity in vitro at dose rates of 1% (Machado et al. 2015b) and 2% (substrate OM basis) in this study. Thus, the inclusion rate for large abatement of CH₄ and degradation of the bioactive metabolite may be managed. However, intensive study of Asparagopsis CH₄ abatement efficacy in cattle and sheep is required.

Reduction effect demonstrated in TGP and CH₄ production at low dose levels of Asparagopsis were not reflected equivalently in the IVD-OM results, which remained unchanged until the 10% dose was used. Inclusion of Asparagopsis had little effect on IVD-OM at dose levels ≤5% of substrate OM, compared with controls (Fig. 3). However, all experiments and studies with Asparagopsis demonstrated a significantly reduced IVD-OM (P < 0.001) at doses of ≥10% (Machado et al. 2014, 2015b). The IVD-OM represented a demonstration of stability in the fermentation and suggests fibre digesting microbes were not affected by Asparagopsis at low dose.

The primary source of energy for ruminant animals is the VFA produced by rumen microbes during digestion of carbohydrates (Bergman 1990) thus negative effects against their production during rumen fermentation is undesirable. The production of TVFA, acetate, and propionate were affected by increasing dose of Asparagopsis. At doses between 1% and 2% the decrease in TVFA was not significant with the HQR substrate used in this study; however, this is not in agreement with a study using LQR where there was a significant decrease in TVFA (Machado et al. 2015b). This may indicate that VFA production may be more sensitive to Asparagopsis in LQR possibly due to higher levels of indigestible fibre and lower protein. However, IVD-OM was similar in both studies and not affected at 1% versus 2% doses, thus the mechanism of the effect is unclear. Generally, TVFA was reduced as the dose level increased. The inclusion of Asparagopsis at low dose levels induced a beneficial change in VFA in favour of propionate as is common with antimethanogenic inclusions. This is believed to be due to competition for the excess hydrogen and reductive propionate production is more favourable than acetogenesis in these conditions (Mitsumori et al. 2012), which may be enhanced by Asparagopsis. It was demonstrated for HQR that significant changes (P < 0.001) in individual VFA concentrations can be achieved. In the present study acetate concentrations decreased and propionate and butyrate increased with increasing doses of Asparagopsis. Changes in acetate and propionate concentrations also reduced the acetate:propionate ratio, which could be partially responsible for corresponding decreases observed...
in CH₄ production in vitro (Beauchemin et al. 2009). The reasoning is that propionate acts as a hydrogen sink; however, production of acetate and butyrate liberates hydrogen thus providing for greater ruminal reduction of CO₂ into CH₄ by methanogens.

Recent studies reporting the antimethanogenic effect of various macroalgae in rumen fermentations has demonstrated variable responses (Dubois et al. 2013; Kinley and Fredeen 2015; Machado et al. 2014). Some macroalgae have previously indicated potential for enteric CH₄ abatement; however, Asparagopsis taxiformis stands out as the most potent. Other macroalgae, particularly the green species appear to be most suitable as novel protein sources with little value as CH₄ abatement agents for ruminants. The production of Asparagopsis at a scale large enough for feeding livestock requires development before commercialisation as a functional feed ingredient. It is unclear how various methods of drying Asparagopsis biomass and subsequent storage will affect levels of secondary metabolites and antimethanogenic potency thus characterisation of the most appropriate methods is required.

Conclusions

A dose of Asparagopsis at 1% of substrate OM exhibited a significant reduction of CH₄ in vitro, and at ≥2% demonstrated virtual elimination of CH₄ with minimal effect on fermentation efficiency of HQR. There was no impact on IVD-OM at dose levels ≤5% and the effect on VFA was a decrease in acetate with a concomitant increase in propionate and to a lesser degree for butyrate. Other areas requiring characterisation is the most appropriate method for processing (dehydration) and feeding to livestock in systems with variable feed quality and content. Nevertheless, using in vitro assessment methods the use of Asparagopsis at low inclusion levels in ruminant diets has demonstrated large CH₄ abatement as a natural product.

References

Kirschke S, Bousquet P, Ciais P, Saunois M, Canadell JG, Dlugokencky EJ, Bergamaschi P, Bergmann D, Blake DR, Bruhwiler L, Cameron-Smith J, Carmichael GF, Ciais P, Christensen TS, Clark DR, Collins L, Con IPPC 150 (2006) Chemical defence against bacteria and antimethanogenic potency thus characterisation of the most suitable as novel protein sources with little value as CH₄ abatement agents for ruminants. Asparagopsis taxiformis biomass and subsequent storage will affect levels of secondary metabolites and antimethanogenic potency thus characterisation of the most appropriate methods is required.

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