

# Dose-response effects of *Asparagopsis taxiformis* and *Oedogonium* sp. on in vitro fermentation and methane production

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**Abstract** This study aimed to identify the optimal doses of the macroalgae *Asparagopsis taxiformis* and *Oedogonium* sp., individually and in combination, which would decrease the in vitro production of methane while minimizing adverse effects on fermentation, using rumen inoculant from *Bos indicus* steers. The dose-response experiment evaluated ten doses of *Asparagopsis* [ranging from 0 to 16.7 % of the organic matter (OM) incubated] and seven doses of *Oedogonium* (ranging from 0 to 100 % OM) using Rhodes grass hay as a basal substrate. *Asparagopsis* was highly effective in decreasing the production of methane with a reduction of 99 % at doses as low as 2 % OM basis. However, a dose of 2 % OM also decreased the production of volatile fatty acids (VFA). *Oedogonium* was less effective with doses  $\geq 50$  % OM significantly decreasing the production of methane. A combination of *Asparagopsis* (2 % OM) and *Oedogonium* (25 and 50 % OM) continued to suppress the production of methane, independent of the inclusion rate of *Oedogonium*. The effectiveness of *Asparagopsis* demonstrates its potential for the mitigation of methane emissions from ruminants at inclusion rates of  $\leq 2$  % OM. *Oedogonium* is a potential feed supplement due to its nutritional value, but supplements  $\leq 25$  % OM are

recommended to avoid adverse effects on apparent in vitro fermentation.

**Keywords** *Asparagopsis* · Rhodophyta · Greenhouse gas mitigation · Methane · Volatile fatty acids · Ruminant · Cattle feed

## Introduction

Animal agriculture, and in particular ruminant enteric methane (CH<sub>4</sub>) production, is the major contributor to greenhouse gas (GHG) emissions from the agricultural sector (Reay et al. 2010). Symbiotic microorganisms in the rumen ferment feed and release energy in the form of metabolic substrate molecules, particularly volatile fatty acids (VFA), which are absorbed by the host ruminant. Concomitantly, methanogens in the rumen microbial consortium use fermentation end-products to generate CH<sub>4</sub> as a hydrogen (H<sub>2</sub>) sink within the rumen (Morgavi et al. 2010). This production of enteric CH<sub>4</sub> has economic costs representing a substantial loss of over 5 % of the total gross dietary energy consumed by the animal (Beauchemin and McGinn 2006; Hristov et al. 2013). Given this loss and the high global warming potential of CH<sub>4</sub>, considerable efforts are being made to develop methodologies to mitigate CH<sub>4</sub> from ruminant production systems.

The management of nutrition in ruminants using traditional or novel plants/forages is a target strategy for the mitigation of CH<sub>4</sub> emissions from livestock (Meale et al. 2012; Patra 2012). This strategy focuses on using the nutritional and biochemical properties of feeds, including secondary metabolites, to manipulate ruminal microbial populations and metabolism to reduce the production of enteric CH<sub>4</sub>, enhance the efficiency of energy use, and consequently the productivity of livestock. Secondary metabolites from terrestrial plants have been

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widely targeted for their antimethanogenic potential (Bodas et al. 2012; Goel and Makkar 2012; Patra 2011). Secondary metabolites may suppress methanogenesis by directly reducing or inhibiting the population of methanogens, and indirectly through the reduction of methanogenic substrate or populations of ruminal protozoa that maintain symbiotic relationships with methanogens (Cieslak et al. 2013). However, high concentrations of secondary metabolites may be required to effectively decrease rumen methanogenesis, which often impairs the fermentation of feed and the overall productivity of the ruminant (Goel and Makkar 2012). Additionally, the effects of secondary metabolites are often variable and contradictory due to the differences in extracts, doses, and the type and quality of basal diet (Martínez-Fernández et al. 2013; Cieslak et al. 2014; Mateos et al. 2013).

Macroalgae and their secondary metabolites have been shown to effectively decrease in vitro methane production (Wang et al. 2008; Machado et al. 2014b; Kinley and Fredeen 2014; Dubois et al. 2013). However, studies to date have been fundamental in their approach utilizing a screening methodology to identify key target species for further investigation. Additionally, the biochemical profile of macroalgae is variable between species and their positive or adverse effects on animal health, and productivity will depend on doses of inclusion in the diet. Of the limited diversity of macroalgae assessed for effects on in vitro methane production, the red macroalga *Asparagopsis taxiformis* shows the greatest potential to inhibit the production of CH<sub>4</sub> (Machado et al. 2014b). This species has high antimicrobial activity, and this may have an effect on the in vitro fermentation of substrates. In contrast, other species of macroalgae have high nutritional value and can improve the production of fermentation products but have lower antimethanogenic potency (Kinley and Fredeen 2014; Machado et al. 2014b; Dubois et al. 2013). Although the antimethanogenic activity of *Oedogonium* is considerably lower than *Asparagopsis*, this green macroalga ameliorates in vitro anaerobic fermentation resulting in higher concentration of metabolizable end-products when added to a low-quality hay, typical of forages available across northern Australia (Machado et al. 2014b).

*Asparagopsis* and *Oedogonium* are two macroalgae with distinct biochemical profiles suitable for use in ruminant production systems. Additional information is required to evaluate the potential of these macroalgae as novel feed supplements for ruminants, namely the antimethanogenic capability and effects on in vitro fermentation. The aim of this study was to evaluate the dose-response effects of *Asparagopsis* and *Oedogonium* individually, and in combination, on the production of CH<sub>4</sub> and main fermentation parameters in vitro. A wide range of doses of each macroalga was assessed to identify an optimal dose to achieve the inhibition of methanogenesis while having minimal adverse effects on overall in vitro fermentation.

## Methods

### Substrates and biochemical analyses

The macroalgae *Asparagopsis taxiformis* (Rhodophyta) and *Oedogonium* sp. [Chlorophyta, Tsv1 GenBank Accession No KC701473 (Lawton et al. 2013)] were acquired from the culture collection of the Centre for Macroalgal Resources and Biotechnology (MACRO) at James Cook University (JCU), Townsville, Australia. Macroalgal biomass was rinsed in freshwater to remove epiphytes, detritus, and sand. The biomass was centrifuged and freeze-dried (VirTis 2 K benchtop freeze-drier) at -55 °C and 120 μbar for 48 h. Rhodes grass hay [*Chloris gayana*, neutral detergent fiber (NDF)= 750 g kg<sup>-1</sup> dry matter (DM); acid detergent fiber (ADF)= 401 g kg<sup>-1</sup> DM] was used as the basal diet of the donor steers and as substrate for in vitro incubations. Freeze-dried macroalgae and air-dried hay samples were ground to 1 mm and stored in airtight containers at -20 °C until incubation.

Dry matter (DM), organic matter (OM), total lipids, carbohydrates, and gross energy (GE) analyses were carried out as previously described by Machado et al. (2014b). Crude protein (CP) fraction was estimated using the total nitrogen content (wt %) of the biomass, which was determined through elemental analysis performed by OEA labs ([www.oelabs.com](http://www.oelabs.com), UK). The CP content was based on the nitrogen factors of 4.7 for *Oedogonium* (Neveux et al. 2014), 4.59 for *Asparagopsis* (Lourenço et al. 2002), and 6.25 for Rhodes grass hay.

### In vitro incubation

Rumen fluid was collected from rumen-fistulated Brahman (*Bos indicus*) steers maintained at the College of Public Health, Medical and Veterinary Sciences, JCU, according to experimental guidelines approved by CSIRO Animal Ethics Committee (A5/2011) and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC 2004). The steers were fed Rhodes grass hay (*Chloris gayana*) ad libitum throughout the study to ensure consistency of microbial activity in the inoculums. Rumen sampling and preparation of rumen-buffer medium were as described by Machado et al. (2014b). In brief, collected rumen fluid was transported in insulated flasks to the laboratory, where they were pooled, blended for 30 s to ensure complete detachment of microbes from the substrate biofilm, and strained through a 0.5-mm mesh under continuous flow of high purity N<sub>2</sub> to maintain anaerobic conditions. Rumen medium was prepared using the strained rumen fluid and a preheated buffer solution (Goering and Van Soest 1970) in a 1:4 (v/v) ratio.

The Rhodes grass hay substrate and macroalgae were weighed into 250-mL Schott bottles, maintaining a total of

1.0 g OM in 125 mL of rumen medium. A blank consisting of the rumen medium only was included in each incubator. Batch culture incubations were conducted using an automated incubation system with RF receivers (Ankom Technology RF, Macedon, NY). Bottles containing substrate, alga, and rumen incubation medium were maintained in dedicated incubators with oscillation platforms (Ratek OM11, Boronia, Australia) at 39 °C and 85 rpm. Each bottle was fitted with an Ankom RF module and monitored for 72 h with gas pressure being measured every 60 s and cumulative pressure recorded every 20 min to generate total gas production (TGP) curves. Incubations were replicated three times, and bottles were randomly allocated between and within incubators.

### Dose-response of individual macroalgae

The effects of *Asparagopsis* and *Oedogonium* were tested individually at a range of doses. Rhodes grass hay was used as a basal substrate to identify the optimum doses of each macroalga for the reduction of methane production while minimizing the adverse effects on anaerobic fermentation. *Asparagopsis* was tested at 0, 0.07, 0.125, 0.25, 0.5, 1, 2, 5, 10, and 16.7 % OM; *Oedogonium* was tested across a broader range of 0, 10, 16.7, 25, 50, 75, and 100 % OM (Table 2), with Rhodes grass constituting the remaining % of OM required to achieve a total 1 g OM incubated. Each treatment consisted of four replicates over three incubation periods. Head space gas samples were collected at 72 h. Apparent degradability of organic matter (OMdeg), production of VFA, which is the main source of energy for the animal (Russell et al. 1992), and pH were also measured at the end of each 72-h incubation period to characterize the effects of macroalgae on in vitro fermentation.

### Dose-response of macroalgae combinations

Based on results of the dose-response effects of macroalgae on fermentation parameters, an experiment was designed to quantify the effects of combining the two macroalgae on VFA production, pH, OMdeg, and total CH<sub>4</sub>. The combinations of *Asparagopsis* (no addition of *Asparagopsis* vs addition of *Asparagopsis* 2 % OM basis) and *Oedogonium* [(0 %, low (25 %), and high (50 % OM)] resulted in six diet treatments, each consisting of a total of four replicates over three incubation periods. Head space gas samples were analyzed for CH<sub>4</sub> at 2, 4, 6, 8, 10, 12, 24, 48, and 72 h. For each treatment, bottles were removed after 72 h to determine the effects on OMdeg and production of VFA.

### Gas and fermentation parameter analysis

Head space gas samples were collected in to pre-evacuated 10-mL vials (Exetainer® vials, Labco Ltd., UK). Head space

gas samples (0.5 mL) were used to determine CH<sub>4</sub> concentrations using gas chromatography (Varian CP-3800), equipped with a BR Q-Plot 30 m×0.53 mm ID column and a flame ionization detector (FID). The injector temperature was 200 °C, column temperature ramped from 65 to 89 °C at a rate of 6.0 °C min<sup>-1</sup> over 4 min. Helium was used as the carrier gas at 2.8 mL min<sup>-1</sup>. Gas standards used for construction of standard curves and quality control during analysis were as described by Machado et al. (2014b). Peak areas were determined by manual integration and CH<sub>4</sub> reported as mL g<sup>-1</sup> OM incubated.

For both experiments (doses of individual and combinations of macroalgae), the fermentation liquor of each replicate was measured for pH, filtered through sintered glass crucibles (porosity 1), and the solid phase dried at 100 °C for 48 h. Organic matter residues of the solid phase were then combusted in a muffle furnace for 8 h at 530 °C for the determination of OMdeg. Filtrates were analyzed for VFA concentrations, which were corrected for the blank as described by Machado et al. (2014b).

### Data analysis

Total gas production (TGP) data were corrected for the blank and fitted to a modified nonlinear sigmoidal model of Gompertz as described by Machado et al. (2014b). The gas production parameters A, B, and C were calculated using a nonlinear procedure (JMP 10, SAS Institute, USA).

Results from the dose-response effects of macroalgae on individual fermentation parameters were analyzed separately for each species of macroalga. One-factor permutational analyses of variance (PERMANOVA) were used to test for significant differences in the mean between doses for each species (fixed factor) on TGP, CH<sub>4</sub> production, VFA, OMdeg, and pH at 72 h of incubation. Pair wise a posteriori comparisons were used ( $\alpha=0.05$ ), where applicable. All analyses were performed using PRIMER 6 [v. 6.1.13; Clarke and Gorley 2006] and PERMANOVA+ [v. 1.0.3; Anderson et al. 2008]. Bray-Curtis similarity matrices were produced using the untransformed raw data, and a dummy variable (0.0001) was used to account for zero values. *P* values of PERMANOVA analyses were calculated from 9999 random permutations of raw data.

Results from the effects of combinations of macroalgae on fermentation parameters were analyzed using two-factor PERMANOVA (Table S1). The effects of the fixed factor dose of *Oedogonium* (0, 25, and 50 % OM) and addition of *Asparagopsis* (0 and 2 % OM) on TGP, CH<sub>4</sub> production, VFA, OMdeg, and pH were tested. Bray-Curtis matrices and *P* values of PERMANOVA analyses were calculated as described above.

**Table 1** Proximate composition of macroalgae and Rhodes grass hay

Substrate	DM g kg <sup>-1</sup> DW	OM	Carbohydrates	CP*	TL	GE (MJ kg <sup>-1</sup> DM)
<i>Oedogonium</i>	939.9	885.6	498.8	228.9	79.4	19.4
<i>Asparagopsis</i>	944.3	936.0	555.8	252.5	33.3	16.8
Rhodes grass hay	902.2	859.4	766.5	66.9	26.0	17.3

Parameters were calculated in g kg<sup>-1</sup> DM, unless otherwise stated. \*Crude protein (CP) fraction was estimated using total nitrogen content (wt %) of the biomass with nitrogen factors of 4.7 for *Oedogonium*, 4.59 for *Asparagopsis*, and 6.25 for Rhodes grass hay

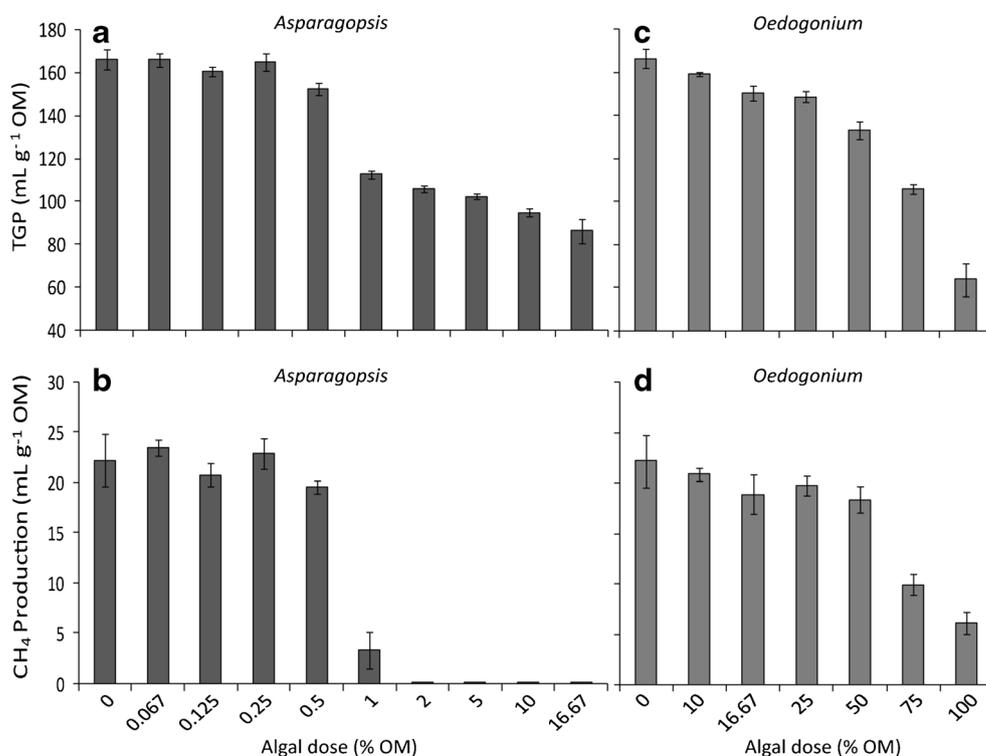
DM dry matter, OM organic matter, CP crude protein, TL total lipids, GE gross energy

## Results

**Biochemical parameters of substrates** Organic matter content varied between substrates and was highest for *Asparagopsis* (936.0 g kg<sup>-1</sup> DM) followed by *Oedogonium* (885.6 g kg<sup>-1</sup>) and was lowest for Rhodes grass hay (859.4 g kg<sup>-1</sup> DM, Table 1). The carbohydrate content of *Oedogonium* was 16 % lower than that of Rhodes grass hay (666.7 g kg<sup>-1</sup> DM), while the carbohydrate content of *Asparagopsis* was similar to that of Rhodes grass hay. The crude protein content of *Asparagopsis* (254.7 g kg<sup>-1</sup>) and *Oedogonium* (231.2 g kg<sup>-1</sup>) was 52.6 and 38.5 % higher than Rhodes grass hay (166.9 g kg<sup>-1</sup> DM), respectively. The lipid contents of *Asparagopsis* (33.3 g kg<sup>-1</sup>) and *Oedogonium* (79.4 g kg<sup>-1</sup>) were 30.4 and 205 % higher than Rhodes grass hay, respectively.

**Dose-response of individual macroalgae** *Asparagopsis* and *Oedogonium* significantly decreased in vitro gas parameters with increasing doses (Fig. 1, Table 2). *Asparagopsis* decreased TGP by 31.5 to 46.5 % compared with the control at doses ranging from 1 to 16.7 % OM (Fig. 1a). The production of CH<sub>4</sub> decreased significantly by 84.7 % at a dose of 1 % OM *Asparagopsis*, and it was virtually eliminated at doses ≥2 % OM, with a decrease of over 99 % compared with the control (Fig. 1b). Although *Oedogonium* was not as effective as *Asparagopsis* in inhibiting methanogenesis, TGP and CH<sub>4</sub> production steadily decreased as the dose of *Oedogonium* increased (Fig. 1c, d). At doses ≥50 % OM, *Oedogonium* significantly decreased TGP by at least 20 % compared with the control (Fig. 1c). At dose ≥75 % OM, *Oedogonium* decreased CH<sub>4</sub> production at least 50 % compared with the control (Fig. 1d), and at a dose of 100 % *Oedogonium*, CH<sub>4</sub> production was reduced by 61.6 %.

**Fig. 1** Dose-response of the macroalgae *Asparagopsis* and *Oedogonium* analyzed individually on the production of total gas (a, c, respectively) and methane (b, d, respectively) after 72 h of in vitro incubation. Note that algal doses (X-axis) vary among species. Error bars represent ±SE (n=4)



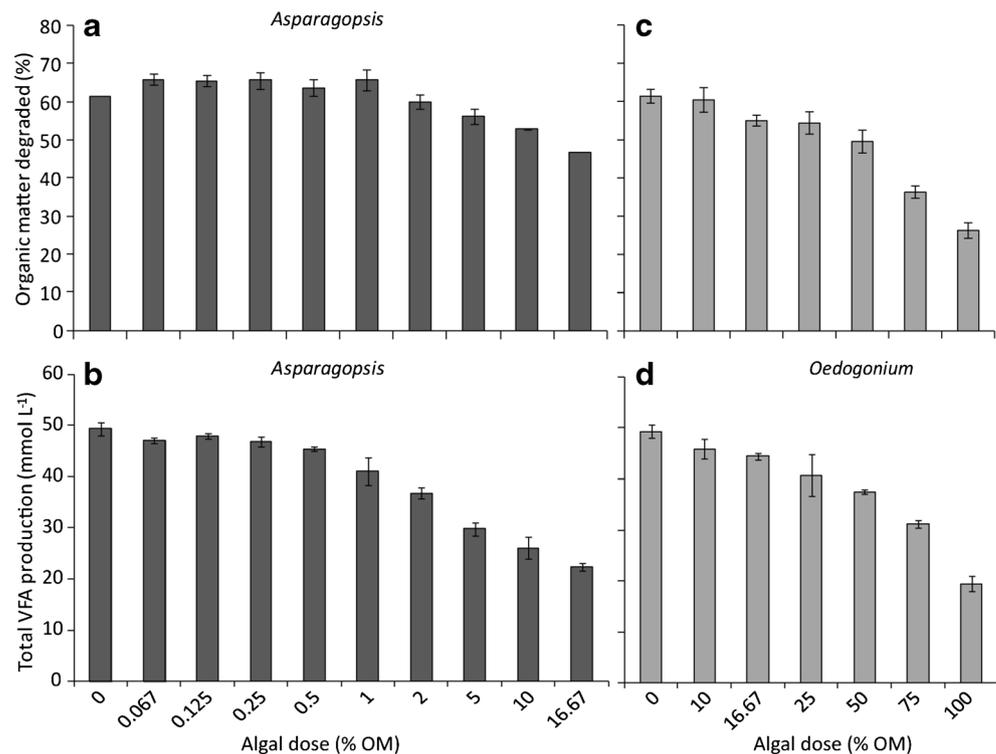
**Table 2** Dose-response of the macroalgae *Asparagopsis* and *Oedogonium* on the in vitro gas and fermentation parameters after 72 h of incubation, and results of one-factor PERMANOVA analyses, with significant effects of algal dose on the different parameters assessed in this study

Species	Algal dose (% OM)	TGP (mL g <sup>-1</sup> OM)	CH <sub>4</sub> (mL g <sup>-1</sup> OM)	OMdeg (%)	Total VFA (mmol L <sup>-1</sup> )	C2 (% Total)	C3	Iso C4	C4	Iso C5	C5	C2/C3 ratio	pH	
<i>Asparagopsis</i>	0	166.3 <sup>a</sup> ±9	22.2 <sup>a</sup> ±4	61.3 <sup>a</sup> ±2	49.3 <sup>a</sup> ±3	66.4 <sup>a</sup> ±2	22.5 <sup>a</sup> ±1	1.6 <sup>b</sup> ±0.2	7.2 <sup>a</sup> ±0.6	1.1 <sup>a</sup> ±0.1	1.2 <sup>a</sup> ±0.2	3.0 <sup>a</sup> ±0.2	6.63	
	0.07	167.2 <sup>a</sup> ±7	23.5 <sup>a</sup> ±2	66.0 <sup>a</sup> ±2	47.1 <sup>ab</sup> ±1	66.1 <sup>a</sup> ±1	22.5 <sup>a</sup> ±1	1.7 <sup>a</sup> ±0.3	7.0 <sup>a</sup> ±0.6	1.3 <sup>a</sup> ±0	1.4 <sup>a</sup> ±0.3	2.9 <sup>ab</sup> ±0.2	6.64	
	0.125	162.2 <sup>a</sup> ±4	20.7 <sup>a</sup> ±2	65.6 <sup>a</sup> ±2	47.9 <sup>ab</sup> ±1	66.6 <sup>a</sup> ±1	22.2 <sup>a</sup> ±1	1.7 <sup>a</sup> ±0.2	6.9 <sup>a</sup> ±0.6	1.2 <sup>a</sup> ±0.1	1.4 <sup>a</sup> ±0.4	3.0 <sup>a</sup> ±0.1	6.64	
	0.25	166.2 <sup>a</sup> ±8	22.9 <sup>a</sup> ±3	65.6 <sup>a</sup> ±2	46.9 <sup>ab</sup> ±2	64.1 <sup>b</sup> ±1	24.1 <sup>b</sup> ±1	1.8 <sup>a</sup> ±0.2	7.4 <sup>a</sup> ±0.8	1.3 <sup>a</sup> ±0.2	1.3 <sup>a</sup> ±0.4	2.7 <sup>b</sup> ±0.1	6.63	
	0.5	153.9 <sup>a</sup> ±6	19.6 <sup>a</sup> ±1	63.7 <sup>a</sup> ±2	45.4 <sup>b</sup> ±1	57.2 <sup>b</sup> ±3	27.9 <sup>c</sup> ±2	1.7 <sup>a</sup> ±0.2	10.0 <sup>b</sup> ±0.7	1.7 <sup>b</sup> ±0.2	1.5 <sup>b</sup> ±0.2	2.1 <sup>c</sup> ±0.3	6.69	
	1	113.9 <sup>b</sup> ±4	3.4 <sup>b</sup> ±3	65.7 <sup>a</sup> ±2	41.1 <sup>bc</sup> ±5	47.4 <sup>d</sup> ±5	33.2 <sup>d</sup> ±3	1.1 <sup>ab</sup> ±0.7	12.8 <sup>c</sup> ±1.5	3.6 <sup>c</sup> ±1.2	1.9 <sup>b</sup> ±0.6	1.4 <sup>d</sup> ±0.2	6.65	
	2	106.7 <sup>c</sup> ±3	<0.05 <sup>Ac</sup>	60.1 <sup>a</sup> ±3	36.8 <sup>c</sup> ±2	41.6 <sup>d</sup> ±2	37.9 <sup>c</sup> ±2	0.4 <sup>bc</sup> ±0.8	15.0 <sup>c</sup> ±1.0	3.1 <sup>bc</sup> ±1.5	2.1 <sup>bc</sup> ±0.7	1.1 <sup>de</sup> ±0.1	6.65	
	5	103.6 <sup>c</sup> ±3	<0.05 <sup>Ac</sup>	56.3 <sup>ab</sup> ±2	29.8 <sup>d</sup> ±3	31.5 <sup>e</sup> ±7	46.8 <sup>f</sup> ±5	0 <sup>Ac</sup>	18.5 <sup>d</sup> ±2.4	0 <sup>Ad</sup>	3.1 <sup>cd</sup> ±0.5	0.7 <sup>e</sup> ±0.3	6.68	
	10	97.0 <sup>d</sup> ±4	<0.05 <sup>Ac</sup>	53.0 <sup>b</sup> ±2	26.1 <sup>de</sup> ±4	29.1 <sup>e</sup> ±8	46.7 <sup>f</sup> ±5	0.5 <sup>ac</sup> ±0.7	19.7 <sup>de</sup> ±2.5	0 <sup>Ad</sup>	4.0 <sup>de</sup> ±1.4	0.6 <sup>e</sup> ±0.3	6.68	
	16.7	88.9 <sup>d</sup> ±11	<0.05 <sup>Ac</sup>	46.8 <sup>c</sup> ±0	22.4 <sup>e</sup> ±2	22.2 <sup>f</sup> ±5	47.4 <sup>f</sup> ±2	0.6 <sup>ac</sup> ±0.9	25.3 <sup>e</sup> ±2.2	0 <sup>Ad</sup>	4.5 <sup>e</sup> ±0.4	0.5 <sup>f</sup> ±0.1	6.73	
	SEM	5.06	1.81	1.12	1.56	2.69	1.69	0.12	1.01	0.21	0.2	0.13	0.01	
	df	9, 30	9, 27	9, 30	9, 30	9, 30	9, 30	9, 30	9, 30	9, 30	9, 30	9, 30	9, 30	9, 30
	Pseudo-F	86.983	63.999	14.099	43.474	32.216	80.408	4.566	83.119	25.047	13.494	40.364	0.683	
	P value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0013	0.0001	0.0001	0.0001	0.0001	NS	
	<i>Oedogonium</i>	0	166.3 <sup>a</sup> ±9	22.2 <sup>a</sup> ±4	61.3 <sup>a</sup> ±2	49.3 <sup>a</sup> ±3	66.4 <sup>a</sup> ±2	22.5 <sup>a</sup> ±1	1.6 <sup>b</sup> ±0.2	7.2±0.6	1.1 <sup>a</sup> ±0.1	1.2±0.2	3.0 <sup>abcd</sup> ±0.2	6.63 <sup>a</sup>
		10	153.5 <sup>b</sup> ±1	20.9 <sup>a</sup> ±1	60.4 <sup>ab</sup> ±3	45.8 <sup>ab</sup> ±4	67.0 <sup>a</sup> ±1	22.3 <sup>a</sup> ±1	1.8 <sup>a</sup> ±0.2	6.2±1.1	1.4 <sup>b</sup> ±0.1	1.4±0.4	3.0 <sup>abc</sup> ±0.3	6.69 <sup>ab</sup>
16.7		150.1 <sup>b</sup> ±7	18.9 <sup>a</sup> ±4	55.0 <sup>b</sup> ±1	44.5 <sup>bc</sup> ±1	65.9 <sup>ab</sup> ±0	22.5 <sup>a</sup> ±0	1.9 <sup>a</sup> ±0.6	7±0.3	1.4 <sup>bc</sup> ±0.2	1.3±0.3	2.9 <sup>ab</sup> ±0.1	6.71 <sup>ab</sup>	
25		148.6 <sup>b</sup> ±5	19.7 <sup>ab</sup> ±2	54.4 <sup>ab</sup> ±3	40.7 <sup>acd</sup> ±8	64.2 <sup>ab</sup> ±6	23.0 <sup>a</sup> ±3	2.1 <sup>ab</sup> ±0.4	7.7±1.4	1.6 <sup>c</sup> ±0.3	1.4±0.7	2.8 <sup>abcd</sup> ±0.6	6.73 <sup>b</sup>	
50		133.2 <sup>c</sup> ±8	18.4 <sup>b</sup> ±3	49.6 <sup>b</sup> ±3	37.5 <sup>d</sup> ±1	67.5 <sup>ab</sup> ±1	21.2 <sup>b</sup> ±1	2.2 <sup>b</sup> ±0.2	6±1.4	1.6 <sup>c</sup> ±0.1	1.4±0.5	3.2 <sup>bc</sup> ±0.1	6.77 <sup>b</sup>	
75		106.1 <sup>d</sup> ±5	10 <sup>c</sup> ±2	36.3 <sup>c</sup> ±2	31.2 <sup>d</sup> ±2	68.5 <sup>b</sup> ±1	20.4 <sup>b</sup> ±2	2.4 <sup>b</sup> ±0.1	6.1±1.2	1.3 <sup>abc</sup> ±0.2	1.2±0.1	3.4 <sup>c</sup> ±0.3	6.93 <sup>c</sup>	
100		63.8 <sup>e</sup> ±15	6.1 <sup>c</sup> ±2	26.2 <sup>d</sup> ±2	19.4 <sup>e</sup> ±3	58.7 <sup>b</sup> ±6	26.1 <sup>ab</sup> ±4	3.7 <sup>c</sup> ±0.6	6.8±1.7	2.1 <sup>abc</sup> ±1	2.6±1.4	2.5 <sup>d</sup> ±0.5	7.01 <sup>c</sup>	
SEM		6.48	1.27	2.46	1.93	0.78	0.48	0.14	0.23	0.09	0.14	0.11	0.03	
df		6, 21	6, 18	6, 21	6, 21	6, 21	6, 21	6, 21	6, 21	6, 21	6, 21	6, 21	6, 21	
Pseudo-F		36.028	14.492	34.272	29.416	3.9757	3.05	18.651	1.071	2.743	2.24	3.4148	11.347	
P-value		0.0001	0.0002	0.0001	0.0001	0.0093	0.0195	0.0001	NS	NS	NS	0.01	0.0002	

Data were analyzed separately for each species of macroalgae; doses not connected by the same letters within the same column for the same species are significantly different ( $\alpha=0.05$ ). Results represent the mean value±SD ( $n=4$ ). Doses not connected by the same letters within the same column for the same species are significantly different

OMdeg organic matter degradability, C2 acetate, C3 propionate, Iso C4 isobutyrate, C4 butyrate, Iso C5 isovalerate, C5 valerate, NS nonsignificant<sup>A</sup> Below detection limits

**Fig. 2** Dose-response of the macroalgae *Asparagopsis* and *Oedogonium* analyzed individually on degradability of organic matter (a, c, respectively) and total production of volatile fatty acids (b, d, respectively) after 72 h of in vitro incubation. Note that algal doses (X-axis) vary among species. Error bars represent  $\pm$ SE ( $n=4$ )



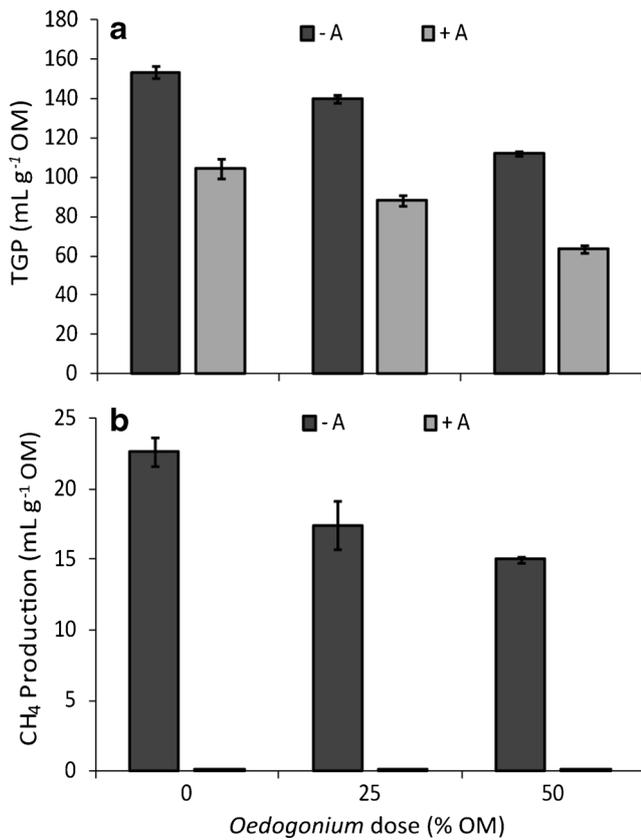
The degradability of organic matter (OMdeg) and the production of total VFA, both measures of efficiency of ruminal fermentation, decreased significantly as the dose of macroalgae increased for both *Asparagopsis* and *Oedogonium* (Fig. 2, Table 2). At doses  $\leq 5$  % OM, *Asparagopsis* had equal or higher OMdeg compared to the control, with OMdeg significantly decreasing at doses  $\geq 10$  % OM (Fig. 2a). Although the degradation of substrate was not affected by the addition of low doses of *Asparagopsis*, the production of total VFA was significantly decreased for doses  $\geq 0.5$  % OM. At doses of 1 and 2 % OM, *Asparagopsis* decreased the concentration of total VFA by 16.6 and 25 %, respectively, compared with the control (Fig. 2b, Table 2), and at doses  $\geq 5$  %, OM total VFA was decreased by 39.5 %. Conversely, the molar proportions of the VFAs propionate, butyrate, valerate, and isovalerate increased significantly for a dose of 2 % OM *Asparagopsis* compared with the control (Table 2) while the proportion of acetate and isobutyrate decreased. Acetate to propionate ratio decreased significantly by 63 % for the 2 % OM dose compared with the control (Table 2).

For *Oedogonium*, OMdeg and total VFA decreased as dose increased, with an average decrease of 11.3 % in OMdeg (Fig. 2c) and 17.4 % in the production of total VFA (Fig. 2d) for the dose of 25 % OM, compared with the control (Table 2). However, only doses of *Oedogonium*  $\geq 50$  % OM significantly decreased OMdeg and VFA compared with the control. The acetate to propionate ratio varied among doses but not from the control (Table 2). Doses of *Oedogonium*

$\geq 25$  % OM significantly increased the pH compared with the control after 72 h (Table 2).

**Dose-response of macroalgae combinations** *Oedogonium* significantly decreased the TGP as the dose increased, and the addition of *Asparagopsis* at 2 % OM further decreased TGP (Fig. 3a, Table 3), showing a significant interaction between dose of *Oedogonium* and addition of *Asparagopsis*. *Oedogonium* alone decreased TGP by 8.5 and 26.9 % at 25 and 50 % OM, respectively, compared with the control (*Oedogonium* 0 % and *Asparagopsis* 0 %). When *Asparagopsis* 2 % OM was added in combination with *Oedogonium* at 25 or 50 % OM, the TGP was decreased by 42 and 59 %, respectively, compared with the control (Fig. 3a). Conversely, *Oedogonium* alone did not affect the production of CH<sub>4</sub> at any dose, and no significant interaction among dose of *Oedogonium* and addition of *Asparagopsis* was detected. The addition of 2 % *Asparagopsis* individually, or in combination with *Oedogonium*, significantly decreased the production of CH<sub>4</sub> by over 99 % independent of *Oedogonium* (Fig. 3b, Table 3).

*Oedogonium* significantly decreased the OMdeg and the production of total VFA as the dose increased (Fig. 4a, Table 3). *Oedogonium* decreased OMdeg by 14 and 24.8 % while VFA was decreased by 10 and 31 %, for the doses 25 and 50 % OM, respectively, compared with the control (*Oedogonium* 0 % and *Asparagopsis* 0 %). The addition of 2 % OM *Asparagopsis* also significantly decreased OMdeg by



**Fig. 3** The effects of combinations of the macroalgae *Asparagopsis* and *Oedogonium* on the production of total gas (a) and methane (b) after 72 h of in vitro incubation. Note that in b, all treatments with addition of *Asparagopsis* fall within the x-axis since the production of methane was near zero or below detection levels. -A, no addition of *Asparagopsis*; +A, addition of 2 % of *Asparagopsis* (OM basis). Error bars represent ±SE (n=4). The control mentioned within the text refers to *Oedogonium* 0 % and no addition of *Asparagopsis*

7 % and production of total VFA by 12 %, compared with the control (Fig. 4, Table 3). When *Asparagopsis* was added at 2 % OM in combination with *Oedogonium* at 25 or 50 % OM, the production of total VFA decreased by 19.6 and 40 %, respectively, compared with the control. However, no significant interaction between dose of *Oedogonium* and the addition of *Asparagopsis* could be detected for OMdeg or total production of VFA. The addition of *Asparagopsis* individually, or in combination with *Oedogonium*, significantly decreased the ratio of acetate to propionate to less than half that of treatments without *Asparagopsis*, independent of the dose of *Oedogonium*.

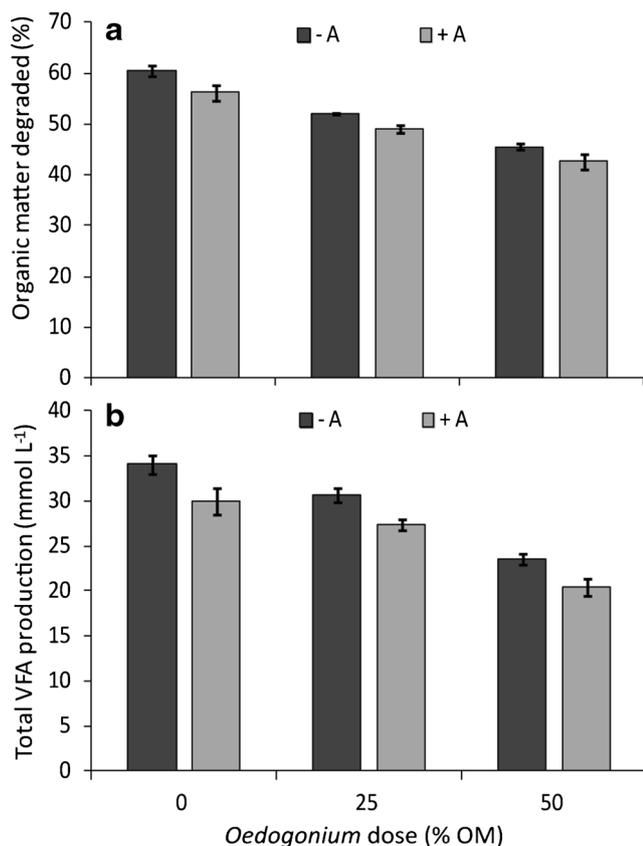
**Discussion**

The effects of *Asparagopsis* and *Oedogonium* on in vitro gas and fermentation parameters are dose-dependent. This study clearly defines the minimum effective dose required to decrease the production of CH<sub>4</sub> in vitro while also identifying

**Table 3** The effects of combinations of the macroalgae *Asparagopsis* and *Oedogonium* on in vitro fermentation parameters after 72 h of incubation and results of two-factor PERMANOVA analyses, with significant effects of dose of *Oedogonium* (DO) and addition of *Asparagopsis* (Ad) on the different parameters assessed in this study

Dose of <i>Oedogonium</i>	Addition of <i>Asparagopsis</i>	TGP (mL g <sup>-1</sup> OM)	CH <sub>4</sub> (mL g <sup>-1</sup> OM)	OMdeg (%)	Total VFA (mmol L <sup>-1</sup> )	C2 (% Total)	C3	Iso C4	C4	Iso C5	C5	C2/C3	pH
0	- A	153.4±3.2	22.7±1.0	60.5±2.1	34.1±2.2	66.8±1.0	22.0±0.8	0.8±0.1	8.2±0.4	1.2±0.1	1.1±0.0	3.0±0.2	6.62±0.04
	+ A	104.4±5.1	0.07 <sup>A</sup>	56.2±3.0	30±2.8	49.5±1.9	34.0±2.9	0.2±0.1	13.5±0.6	1.5±1.7	1.3±0.1	1.5±0.2	6.63±0.05
25	- A	140.3±2.0	17.4±1.7	52.0±1.3	30.6±1.7	66.6±0.6	22.0±0.6	0.9±0.0	7.9±0.3	1.4±0.2	1.1±0.0	3.0±0.1	6.68±0.04
	+ A	88.2±2.5	0.05 <sup>A</sup>	49.1±1.3	27.4±1.2	48.2±2.3	35.3±3.0	0.4±0.1	13.9±0.7	1.0±1.3	1.3±0.1	1.4±0.1	6.70±0.03
50	- A	112.2±0.9	15.0±0.2	45.5±1.1	23.5±1.2	66.2±1.8	22.7±1.6	1.0±0.1	7.6±0.6	1.3±0.4	1.2±0.0	2.9±0.3	6.77±0.04
	+ A	63.6±2.0	0.06 <sup>A</sup>	42.7±2.5	20.4±2.0	40.4±3.2	40.3±3.1	0.6±0.1	16.1±1.0	1.0±1.3	1.6±0.2	1.0±0.1	6.78±0.04
	DO	0.0048	NS	0.0001	0.0001	0.0006	0.0249	0.0002	NS	NS	0.0006	0.0038	0.0005
	Ad	0.0001	0.0001	0.0011	0.0008	0.0001	0.0002	0.0001	0.0001	0.0013	0.0001	0.0001	NS
	DO x Ad	0.0002	NS	NS	NS	0.0007	NS	0.0002	0.0037	NS	NS	0.009	NS

Control, 100 % Rhodes grass hay; -A, no addition of *Asparagopsis*; +A, addition of 2 % of *Asparagopsis* (OM basis) (±SD, n=4). Pseudo-F values are given in Table S1  
 OMdeg organic matter degradability, C2 acetate, C3 propionate, Iso C4 isobutyrate, C4 butyrate, Iso C5 isovalerate, C5 valerate



**Fig. 4** The effects of combinations of the macroalgae *Asparagopsis* and *Oedogonium* on degradability of organic matter (a) and total production of volatile fatty acids (b) after 72 h of in vitro incubation. Note that in b, all treatments with addition of *Asparagopsis* fall within the x-axis since the production of methane was near zero or below detection levels. -A, no addition of *Asparagopsis*; +A, addition of 2 % of *Asparagopsis* (OM basis). Error bars represent  $\pm$ SE (n=4). The control mentioned within the text refers to *Oedogonium* 0 % and no addition of *Asparagopsis*

the doses at which the production of fermentation products, and potentially animal production, is affected. *Asparagopsis* has potent antimethanogenic activity with doses as low as 2 % OM decreasing the production of CH<sub>4</sub> by more than 99 %, providing similar levels of reduction to that reported for other potent antimethanogens such as bromochloromethane (Goel et al. 2009) and 2-nitroethanol (Zhou et al. 2011). *Asparagopsis* produces more than 100 low molecular weight metabolites containing bromine, iodine, and chlorine that have antimicrobial activity (Woolard et al. 1979; Paul et al. 2006). Notably, bromoform is the most abundant metabolite produced by *Asparagopsis* (Paul et al. 2006). Halogenated analogs are known to inhibit methanogenesis by reacting with a vitamin B<sub>12</sub> cofactor, thereby inhibiting the enzymatic reaction that decreases cobamide-dependent methane formation (Wood et al. 1968). Similar halogenated compounds have demonstrated long-lasting effects on methanogenesis in vivo with limited effects on animal production (Tomkins et al. 2009).

The organic matter ingested by ruminants is degraded through anaerobic fermentation by the rumen microbial

consortium generating VFA, the main source of energy for ruminants (Russell et al. 1992), and both OMdeg and production of VFA are indicators of fermentation efficiency. In this study, OMdeg and the production of total VFA decreased as the dose of macroalgae increased for both *Asparagopsis* and *Oedogonium*. However, only doses of *Asparagopsis*  $\geq$ 10 % OM significantly affected the OMdeg. Nevertheless, the final concentration of total VFA decreased by 12 to 25 % at a dose of 2 % OM (see Tables 2 and 3), due to a decrease in the production of acetate lowering the ratio of acetate to propionate. Nevertheless, the proportion of propionate, butyrate, valerate, and isovalerate increased significantly for the *Asparagopsis* dose of 2 % OM, suggesting that alternative fermentation processes take place when methanogenesis is inhibited. The absence of significant detrimental effects on in vitro fermentation parameters, in particular the OMdeg, using lower doses (<5 % OM) of *Asparagopsis* supports the potential for the macroalga to decrease the CH<sub>4</sub> production with minimized adverse effects on ruminal fermentation. The use of extracts or purified metabolites from *Asparagopsis* may further decrease any impact on the production of VFA while maintaining antimethanogenic bioactivity. Consequently, the next challenge in understanding the mechanism of action of *Asparagopsis* is the quantitative and qualitative analysis of the effects of specific secondary metabolites produced by this alga on the diversity and activity of the rumen microbial biome.

Although *Oedogonium* was not as effective as *Asparagopsis* in inhibiting methanogenesis, *Oedogonium* significantly decreased the production of CH<sub>4</sub> as the dose increased. Nevertheless, in this study, *Oedogonium* was less effective in decreasing the in vitro production of CH<sub>4</sub> than reported in previous studies (Dubois et al. 2013; Machado et al. 2014b). Differences in substrate used across these studies may have contributed to the variable antimethanogenic response. In general, substrates with a high protein content generate lower volumes of total gas on a per gram of OM basis (Cone and van Gelder 1999) and methane (Johnson and Johnson 1995) than fibrous low-quality material. The *Oedogonium* used in this study had higher gross energy and lipid content and 3.4 times higher crude protein content than Rhodes grass hay (Table 1). The inclusion of lipids in ruminant diets has also been demonstrated to decrease methanogenesis in cattle (Beauchemin et al. 2007). The decrease in TGP and production of CH<sub>4</sub> may be related to the protein and lipid content of *Oedogonium*, especially when included at higher doses.

The OMdeg and the production of VFA were significantly decreased for doses of *Oedogonium*  $\geq$ 50 % OM. The production of VFA is related primarily to the availability of carbohydrates in the rumen (France and Dijkstra 2005). The increased lipid and protein content associated with the higher doses of *Oedogonium* decrease the proportion of carbohydrates

available for the production of VFA thereby lowering the concentration within the incubations. Nevertheless, *Oedogonium* has the potential to improve the production of VFA in environments characteristic of northern Australia, where dietary crude protein is a limiting nutrient for the production of rumen microbial crude protein (Poppi and McLennan 1995), particularly during the dry season (Machado et al. 2014a). *Oedogonium* has the potential to be included in intensive beef cattle production systems as an alternative energy source where algal biomass could be cultured using wastewater (Cole et al. 2015; Hu et al. 2013; Chen et al. 2012). This offers a potentially sustainable and novel feed source to the livestock industry. Additionally, *Oedogonium* has a high content of polyunsaturated fatty acids (PUFA (Machado et al. 2014b)), with health benefits for ruminants and resulting in improved meat quality (Scollan et al. 2006).

The effectiveness of commonly used feed additives in decreasing the production of CH<sub>4</sub> typically varies with the type and quality of substrate (O'Brien et al. 2014). In this study, the addition of 2 % *Asparagopsis* eliminated the production of CH<sub>4</sub> independent of the dose of *Oedogonium*. The results are consistent with previous halogenated CH<sub>4</sub> inhibitors tested in vitro (O'Brien et al. 2014; Lee et al. 2009). Halogenated analogs, such as those found in *Asparagopsis*, are effective in decreasing the in vitro methanogenesis independent of the type and quality of the basal substrate used here (Rhodes grass only or in combination with *Oedogonium*). The combination of *Asparagopsis* and *Oedogonium* had a cumulative effect on fermentation parameters, indicating that the type and quality of substrates influence the extent of the adverse effects on in vitro fermentation.

In conclusion, this study demonstrated that *Asparagopsis* is a potent antimethanogen as the optimal dose of 2 % OM decreased the in vitro production of CH<sub>4</sub> by over 99 % compared with the control. At this low dose, negative effects on fermentation parameters are minimized. Therefore, *Asparagopsis* is a potential feed additive for CH<sub>4</sub> mitigation in ruminant production systems. *Oedogonium* was a less potent antimethanogenic agent, but its nutritional value indicates that it could be used as a feed supplement at levels of ≤25 % of the diet (OM).

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