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New temperate seaweed targets for mitigation of ruminant methane emissions: an in vitro assessment

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ABSTRACT

Methane is a potent greenhouse gas with a comparatively short (9 yr) atmospheric lifetime; therefore effective strategies for methane mitigation will contribute significantly to meeting the UN Sustainable Development Goal #13 of taking rapid action against climate change. Methane emissions from enteric fermentation constitute a large proportion of agricultural greenhouse gas emissions. Low inclusions of red seaweed from the genus *Asparagopsis* have demonstrated near elimination of enteric methane from ruminants; however, only a limited number of other seaweeds have been assessed for their anti-methanogenic potential. New Zealand red seaweed species *Bonnemaisonia hamifera, Euptilota formisissima, Plocamium cirrhosum, Vidalia colensoi*, and identified aquaculture target species *Ecklonia radiata* and *Ulva* sp. B were investigated as anti-methanogenic feed additives. Seaweeds were included at 0%, 2%, 6%, or 10% of feed organic matter (OM, ryegrass hay) during *in vitro* fermentation assays using rumen inoculant from non-lactating Friesian x Jersey dairy cows, using *Asparagopsis armata* as a positive control. Total gas, methane, hydrogen, volatile fatty acids, and organic matter degradation were measured over a 48 h incubation. Inclusion of all seaweeds except *Ulva* sp. B reduced the production of methane at either 6 or 10% OM. *Bonnemaisonia hamifera* was the best performing seaweed, reducing the production of methane by 17.1%, 95.4%, and 98.8% relative to the basal feed substrate control at inclusion levels of 2%, 6%, and 10% OM, respectively, with notable increases in the production of hydrogen. *Euptilota formisissima* and *P. cirrhosum* reduced the production of methane by up to 50.5 and 39.5%, respectively, at an inclusion level of 10%, with minimal effects on measured fermentation parameters. Bromoform, the primary bioactive component in *Asparagopsis*, was not detected in any of the new seaweeds tested. Our results therefore identify potential alternative anti-methanogenic seaweed targets that are less susceptible to the loss of volatile bioactives during processing.

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Introduction

Methane (CH_4) is a potent greenhouse gas (GHG) contributing to global climate change and has a high global warming potential (GWP) (Myhre et al., 2013). While CH_4 has a relatively short atmospheric life time (~9 years) (Prather et al., 2012), its emissions make up approximately 20% of global GHG emissions (Olivier et al., 2019). Agriculture is the primary source of global anthropogenic CH_4 emissions (41%) (ClimateWatch, 2020), which are dominated by emissions from ruminant production systems, *i.e.*, CH_4 derived from enteric fermentation (17% of global CH_4 emissions) (Knapp et al., 2014; Saunois et al., 2020). In contrast, agriculture contributes to 85% of New Zealand's CH_4 emissions (ClimateWatch, 2020), with enteric fermentation contributing 74.1% (Ministry for the Environment, 1986). Thus, the development of strategies to mitigate CH_4 emissions from ruminant production systems is critical for increasing the sustainability of these industries, especially in the context of New Zealand. Furthermore, the high global warming potential and short atmospheric life time of CH_4 means that the implementation of effective solutions for CH_4 mitigation will have rapid impacts, and therefore contribute substantially to meeting the UN Sustainable Development Goal #13 of taking urgent and decisive action against climate change (United Nations, 2016).

Strategies to reduce CH_4 emissions from enteric fermentation include legislation (Key & Tallard, 2012), selective breeding (Basarab et al., 2013), antibiotics (Grainger et al., 2008), nutritional strategies (Beauchemin et al., 2008; Hristov et al., 2009), feed

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additives (Bayat et al., 2018; Durmic et al., 2014; Hristov et al., 2009; Machado et al., 2014; Melgar et al., 2016), and vaccines (Wedlock et al., 2010; Williams et al., 2005). These strategies reduce CH_4 production to various degrees, ranging from 12% with the addition of antibiotics, to up to 98% with the inclusion of select feed additives (Chagas et al., 2019; Hristov et al., 2015; Kinley et al., 2020; Martinez-Fernandez et al., 2017; Roque et al., 2012; Tomkins et al., 2009). In this respect, recent research has demonstrated that seaweeds rich in bioactive secondary metabolites are among the most effective feed additives for mitigating enteric CH_4 emissions (Choi et al., 2021; Dubois et al., 2013; Machado et al., 2014). To date, seaweed species from the genus *Asparagopsis* are the most effective additives, reducing the production of enteric CH_4 by 98% at low inclusion rates (e.g., 0.2–2% of feed organic matter (OM)) (Kinley et al., 2016; Kinley et al., 2020; Li et al., 2016; Machado et al., 2016; Roque et al., 2012). However, only a limited number of seaweeds have been assessed for their capacity to mitigate enteric CH_4 emissions.

Over 900 species of seaweeds are currently recognized throughout New Zealand (Nelson et al., 2019), yet only a fraction of these species have been tested for their capacity to mitigate enteric CH_4 production (Machado et al., 2014; Maia et al., 2016). Species of brown seaweed including *Cystoseira trinodis* and *Dicotya bartayresii* and species of green seaweed from the genus *Ulva* have demonstrated significant anti-methanogenic activity *in vitro* and reduced total gas production (Dubois et al., 2013; Machado et al., 2014). However, aside from *Asparagopsis*, the anti-methanogenic potential of red seaweeds remains largely untested. Red seaweeds produce a large variety of halogenated secondary metabolites (Kladi et al., 2004), some of which are likely candidates for exhibiting anti-methanogenic effects. Therefore, this study investigated the anti-methanogenic properties of New Zealand seaweeds that included examples of red (*Bonnemaisonia hamifera, Euptilota formisissima, Plocamium cirrhosum, Vidalia colensoi*), brown (*Ecklonia radiata*), and green (*Ulva* sp. B) seaweeds. *Ecklonia radiata* and *Ulva* sp. B were chosen specifically as there is an opportunity for rapid development and industry implementation due to both existing and developing aquaculture for securing supply, which aligns with meeting the UN Sustainable Development Goal #13 of taking rapid action against climate change (United Nations, 2015). We quantified the anti-methanogenic activity of seaweeds using *in vitro* fermentation assays, and measured changes in hydrogen (H_2), volatile fatty acids (VFAs), and organic matter degradation (OMdeg). The anti-methanogenic activity was benchmarked against *Asparagopsis armata*.

Methods and materials

Sample collection

Samples of seven species of seaweed were collected from natural populations (Ministry for Primary Industries Special Permit number 560) or stock cultures. Bulk material (approximately 50 g fresh weight) of *A. armata, B. hamifera, E. formisissima, P. cirrhosum, Vidalia colensoi* (all gametophytes) and *Ecklonia radiata* (sporophytes) were collected from intertidal rocky shore or reef habitats from six sites located in the North Island, New Zealand (Table 1). Samples of *A. armata* and *E. radiata* were identified using morphological characteristics (Nelson, 2020). *Bonnemaisonia hamifera, P. cirrhosum* and *E. formisissima* were identified using DNA barcoding. DNA was extracted from 20 mg dried tissue of each sample using the CTAB method of Zuccarello & Lokhorst (2005). The *rbc*L locus was amplified and sequenced using various combinations of primers F57, F145, F762, R753, RrSs, R898, and R1442 (Nelson et al., 2010; Freshwater & Rueness, 1994). Sequences were trimmed and assembled using Geneious Prime 2020.2.3, and the consensus sequences were compared with sequences in GenBank using BLAST https://blast.ncbi.nlm.nih.gov/). DNA sequences were submitted to Genbank under the following accession numbers: *B. hamifera* – MZ604718, *P. cirrhosum* – MZ604717, and *E. formisissima* – MZ604719. *Ulva sp. B* (Genbank accession number MW250819.1) was sampled from a mixed gametophyte/sporophyte stock culture cultivated at the Coastal Marine Field Station, University of Waikato, Tauranga, in nutrient enriched (Cell-Hi F2P, Varicon Aqua Solutions UK, 0.1 g l^{-1}, 12.3 mg nitrate-N l^{-1} and 1.1 mg P – [1]) filtered seawater, under a 12/12 h light/dark cycle at 18°C (Lawton et al., 2021). Wild harvested material was chilled on ice for transportation back to the laboratory

Table 1. Collection sites of seaweed specimens used for this study. All locations were within the North Island, New Zealand.

Species	Collection site	GPS co-ordinates	Collection date	Depth (m)
Asparagopsis armata	Mathesons Bay	36.31°S, 174.80°E	Oct 2019	2–3
Bonnemaisonia hamifera	Mathesons Bay	36.31°S, 174.80°E	Oct 2019	2–3
Euptilota formisissima	Tauranga harbour	37.60°S, 176.05°E	Dec 2019	4–7
Plocamium cirrhosum	Makara Beach	41.22°S, 174.71°E	Jan 2020	6–7
Vidalia colensoi	Papatea Bay	37.64°S, 177.84°E	Nov 2019	1–2
Ecklonia radiata	Motuotau Island	41.27°S, 173.14°E	Dec 2019	3–5
Ulva species B (WELT A027378; sp. 1 sensu Heesch et al., 2009)[1]	Cultivated biomass	37.60°S, 176.05°E	Dec 2019	N/A

[1]Nelson et al., 2019.

prior to being frozen and stored at –20°C. All samples were then freeze-dried (48 h, 50 mBar, Buchi L-200 Freeze Dryer, OneLab, New Zealand) and milled to a fine powder using a domestic blender and stored at –80 °C until use.

Compositional analysis

Elemental analysis (% carbon, hydrogen, nitrogen, sulphur, bromine, chlorine, iodine; n= 2 as subsamples of homogenized biomass) for each species was determined through percentage elemental analyses performed by OEA labs (www.oealabs.com, Callington, UK), where carbon, hydrogen, nitrogen and sulphur were determined using gas chromatography-thermal conductivity detector (GC-TCD), and chlorine, bromine, and iodine were determined using ion chromatography (IC). The content of crude protein (CP) was estimated using the total content of nitrogen (wt%) in the biomass with nitrogen-protein conversion factors of 5.63 for *Asparagopsis*, 5.10 for remaining red species of seaweed, 4.49 for *E. radiata*, 5.14 for *Ulva* sp. B, and 6.25 for perennial ryegrass (RG299, basal feed substrate) (Angell et al., 2016). Analyses of crude fat (CF, AOCS 1 Official Procedure AM-5-04), acid detergent fibre (ADF, AFIA Method 1.9A(a)), neutral detergent fibre (NDF, NFTA method adapted for Ankom autoanalyser), soluble sugars (80:20 Ethanol:Water extraction and colorimetric determination, in house method), and starch content (Enzymic Hydrolysis of Starch, colorimetric determination of glucose, in house method) for seaweed and RG299 were performed by R J Hills Laboratories Limited (www.hills-laboratories.com), New Zealand. Results are reported "as received", *i.e.*, not corrected for residual moisture (typically 5%), except for the content of CP for RG299. Polyphenols were quantified following Zhang et al. (2006) modified as per Magnusson et al. (2015) and scaled up for the use of cuvettes.

Quantification of bromoform

Bromoform content was analysed (n = 3 as subsamples of homogenized biomass) in all species as bromoform has been identified as the main anti-methanogenic bioactive in *Asparagopsis* spp. (Machado et al., 2016a). Dried milled biomass (~100 ± 0.001 mg) in methanol (10 ml; HPLC grade, Fisher Chemical) was vortexed (10 sec) followed by sonication (30 min at <10°C; XUB5, Grant Instruments) and then centrifuged at 3200 g (10 min at 4°C; 1248 R, LabTech). The extraction process was repeated using the same biomass, and the two methanol extracts were combined (20 ml). The combined extract was then diluted (1:100) with methanol (HPLC grade, Fisher Chemical) and a 1 ml aliquot was then transferred into a 2 ml amber glass vial in preparation for gas-chromatography mass-spectrometry (GC-MS) analysis. Samples were analysed in scan mode by GC-MS (Shimadzu GC-2030 with GCMS-QP 2020 NX fitted with a Shimadzu 30 m SH-Stabilwax column (221–75,972-30)) using 1 µl injections, pulsed (9.8 psi) split-less mode, with temperatures of the injection port (180°C), transfer line (280°C), and oven (held at 40°C for 1 min, ramped at 16°C min^{-1} to 250°C, then held at 250°C for 2 min) using He (0 grade, BOC) purge gas at 4 ml min^{-1}. Bromoform was identified by its characteristic ion fragments (m/z: 170.8, 174.8, 251.8, 253.8) and quantified by comparison with a standard curve of bromoform (0.025–5.0 µg ml^{-1}) using certified reference material (36,972, Supelco).

In vitro fermentation assay

The *in vitro* fermentations were conducted in three separate incubation runs using a published method (Muetzel et al., 2014). Briefly, 500 ± 15 mg of air-dried feed substrate perennial ryegrass (basal feed substrate) with seaweed biomass (*B. hamifera, E. formisissima, P. cirrhosum, V. colensoi, E. radiata*, or *Ulva* sp. B) included at 2%, 6%, or 10% OM on top of the basal feed substrate was weighed into 125 ml incubation bottles and covered with Parafilm until use. The inclusion level of 2% OM was selected as we expect a highly effective species to have a strong effect here, and a moderately effective species to exhibit some effect. We then evenly spread (increased by 4%-unit steps) the inclusion level up to 10% OM, as inclusion levels above this dose require amounts of seaweed that become impractical and prohibitive to implement for large-scale cattle herds. For the positive control, *A. armata* was added at a dose of 2% OM and a sample without added seaweed served as the negative control. A standard ryegrass used in every incubation was used as a run control to identify low activity incubations (Muetzel et al., 2014). Each of these treatments was incubated in two bottles (technical replicates). The prepared incubation bottles were randomized and pre-warmed in a 39°C incubator prior to incubation. The rumen fluid donor animals were maintained according to the guidelines approved by the AgResearch Animal Ethics committee (AE 15320). Rumen fluid from a two non-lactating fistulated Friesian x Jersey dairy cows was collected into pre-warmed thermos flasks and filtered through one layer of cheesecloth and equal volumes were added at a level of 20% v/v to the 3.2 l of pre-warmed (39°C) *in vitro* buffer (6.0 mM Na_2HPO_4, 9.6 mM KH_2PO_4, 0.5 mM $MgCl_2$, 64.5 mM $NaHCO_3$ and 17.8 mM NH_4HCO_3 (Mould et al., 2005)). The medium was dispensed in 50 ml aliquots under a stream of CO_2 into the prepared incubation bottles, which were then capped with a butyl rubber stopper and placed in a rack in an incubator at 39°C. The bottles were connected to the gas system by perforation

of the butyl rubber stopper with a syringe needle (23 g) connected to the gas system. The bottles were constantly shaken on a reciprocal shaker at 120 rpm and incubated for 48 h. For statistical analysis the incubation was repeated three times using rumen fluid from different donor animals. Technical replicates for each incubation were averaged and included in the final dataset. Results from the three incubations were analysed as a single dataset as there was no significant difference in TGP between the run controls (Supplementary figure S1).

In vitro fermentation with rumen fluid

Gas accumulation was measured automatically using a pressure sensor, described in Muetzel et al. (2014). Once a bottle had reached the threshold pressure of 9 kPa above ambient pressure, the gases were released into a gas chromatograph (GC-2010, Shimadzu, Kyoto, Japan) via a 6-port valve and a 20 µl sample loop. The GC was fitted with a HP-MolseivePlot column (30 m length × 0.53 mm ID), a thermal conductivity detector (TCD) and flame ionization detector (FID) (maintained at 105°C and 250°C, respectively) in series to simultaneously quantify CH_4 and H_2 production from each incubation bottle. The column temperature was maintained at 85°C using N_2 as a carrier with a flow rate of 13 ml/min. Each GC is attached to an array of 36 bottles and has a maximum analysis frequency of one sample per minute. Fermentation gases were quantified using a calibration of four standards containing 1, 5, 10, 20% CH_4 and 0.5, 2.5, 5, 10% H_2 in nitrogen respectively (BOC Gases New Zealand Ltd. (Muetzel et al., 2014)).

Samples for VFA analysis (1.8 ml bottle) were collected from each incubation bottle after 48 h using a wide bore tip. Samples were centrifuged (21,000 × g, 10 min, 4°C) and 900 µl was combined with 100 µl of internal standard solution (19 mM ethylbutyrate in 20% v/v phosphoric acid). The samples were stored at – 20°C for at least 16 h, thawed, and centrifuged again as described above. An 800 µl aliquot of the combined supernatant and internal standard solution was transferred into a 2 ml crimp gas chromatography vial and crimped immediately. VFAs were analysed by gas chromatography as described by Attwood et al., (1998) in a Shimadzu GC2010plus gas chromatograph equipped with a flame ionization detector using a Zebron ZB-FFAP 30.0 m × 0.53 mm I.D. × 1 µm film column (Tavendale et al., 2005) and an FID detector.

The degradability of organic matter (*OMdeg*, % degraded) was calculated using the equation

$$OMdeg = 14.88 + 0.889\ GP + 0.045\ CP + 0.065\ ASH$$

where *GP* is the volume of gas (ml 200 mg^{-1}) produced at 24 h (obtained from *in vitro* incubations), and *CP* and *ASH* are the total crude protein and ash content (mg g^{-1} dry weight (DW)), respectively, of substrate(s) used for *in vitro* incubations (Menke & Close, 1986).

Statistical analyses

Prior to data analyses, gas production data were checked for errors (*i.e.*, system calibration errors and gas leaks) during the *in vitro* incubations, and bottles identified as errors (1x *B. hamifera* 6%, 1x *D. compressa* 2%, and 1x *A. armata* 2%) were excluded from analyses. The effect of seaweed inclusion level (fixed factor) on total gas, CH_4, H_2, total VFA, and individual VFA production and OMdeg was analysed using permutational analyses of variance (PERMANOVA) conducted in Primer v7 (Primer-E Ltd., UK) using Euclidean distances resemblance matrices, 9,999 unrestricted permutations of raw data, and Type III sum of squares (Anderson et al., 2008). Data for each species were analysed separately and compared to the ASP 2% OM positive control and the basal feed substrate control using planned contrasts. Monte Carlo *P*-values were used to assess significance (Anderson et al., 2008). Correlations between variables were examined using Pearson's correlation coefficients conducted in MS Excel (Version 1808).

Results and discussion

The inclusion of New Zealand native seaweeds as feed additives reduced the *in vitro* production of CH_4 in rumen fluid by up to 99% relative to the basal feed substrate control (Fig 1), with significant changes in other measures (H_2, VFAs, and OMdeg) that are used to assess the efficiency of rumen fermentations. Bromoform, the main bioactive in *Asparagopsis* that inhibits methanogenesis, was not detected in the new seaweeds tested (Table 2), indicating that the bioactivity originates from alternative anti-methanogenic compounds.

Compositional analysis

The use of seaweed secondary metabolites as potential feed additives to reduce enteric CH_4 emissions has recently attracted much research interest, especially due to a growing preference for natural additives over antibiotics and/or chemical additives (Clark et al., 2010; Kobayashi, 2010). Many seaweed secondary metabolites are halogenated compounds and their presence are reflected in higher tissue contents of halides (e.g., bromine, chlorine, iodine).

In the current study, the organic matter content of seven seaweeds varied widely (44–80% DW; Table 3) while ash content was highly correlated with total halogen content (R^2 = 0.85) and chlorine content (R^2 = 0.88). Only a weak correlation was observed for bromine (R^2 = 0.24) and iodine (R^2 = 0.36), consistent with the differential tissue accumulation of bromine and/or iodine in the seaweeds studied here. *A. armata* had the highest content of bromine (7.1%) which was approximately double that of *V. colensoi* (3.9%) and four times that of *B. hamifera* (1.6%) (Table 2); this is consistent with the characteristic brominated secondary metabolites reported for these species (Table S1). The content of bromoform, the main anti-methanogenic compound in *A. armata*, was 10.4 mg/g DW; which is similar to that measured for high-quality (6.6–7.8 mg g^{-1} DW) *Asparagopsis* spp. previously (Kinley et al., 2020; Roque et al., 2021). Despite the relatively high contents of bromine in *V. colensoi* and *B. hamifera*, bromoform was not detected in these or the other seaweeds tested in the current work, however these species contain other brominated compounds (Supplementary table S1). The iodine content of *A. armata* (1.0%), *B. hamifera* (0.8%), *E. radiata* (0.4%) and *P. cirrhosum* (0.1%) illustrate the need for measurement of this element for seaweed biomasses targeted at animal feed inclusions. While iodine is an essential element critical for animal function, and it is recognized that New Zealand cattle populations are commonly deficient in iodine (Anderson et al., 2007), excessive doses (tolerable upper limit for cattle: 50 mg iodine kg^{-1} dry matter (DM) day^{-1} (Weiss, 2005)) can result in negative effects on animal health and production (National Academies of Sciences, Engineering, and Medicine, 2016; Paulíková et al., 2002; Weiss, 2005). Polyphenols ranged from 2.1 to 55.3 mg g^{-1} DW and were highest for *E. radiata* (Table 3). Polyphenols can act as a H_2 sink during enteric fermentation highlighting the potential for producing a combination seaweed supplement to mitigate CH_4 emissions while simultaneously alleviating the increased partial pressure of H_2 in the rumen (Martinez-Fernandez et al., 2017).

Table 2. Elemental composition (wt%) of carbon (C), hydrogen (H), nitrogen (N), sulphur (S), bromine (Br), chlorine (Cl), and iodine (I), and concentration (mg/g DW) of bromoform (BF) for seaweed species *Asparagopsis armata* (ASP, positive control), *Bonnemaisonia hamifera* (BNM), *Euptilota formisissima* (EPT), *Plocamium cirrhosum* (PLC), *Vidalia colensoi* (VDA), *Ecklonia radiata* (ECK), and *Ulva* sp. B (ULVA).

Seaweed	C	H	N	S	Br	Cl	I	BF
ASP	19.5	3.0	2.7	2.8	7.1	14.8	1.0	10.4
BNM	20.8	3.5	2.3	3.0	1.6	17.0	0.8	–
EPT	17.0	3.0	2.6	2.4	0.7	13.3	0.0	–
PLC	25.1	4.0	3.5	5.1	1.3	9.5	0.1	–
VDA	32.6	4.7	2.7	3.0	3.9	5.9	0.0	–
ECK	32.3	5.0	1.6	1.2	0.1	8.0	0.4	–
ULVA	33.8	4.0	3.7	3.4	0.1	4.4	0.0	–

Table 3. Composition (%DW) of organic matter (OM), ash, crude protein (CP), crude fat (CF), acid detergent fibre (ADF), neutral detergent fibre (NDF), soluble sugars, starch, and polyphenols (PP) (mg/g DW) for perennial ryegrass (negative control), and seaweed species *Asparagopsis armata* (ASP, positive control), *Bonnemaisonia hamifera* (BNM), *Euptilota formisissima* (EPT), *Plocamium cirrhosum* (PLC), *Vidalia colensoi* (VDA), *Ecklonia radiata* (ECK), and *Ulva* sp. B (ULVA).

Seaweed	OM	ASH	CP	CF	ADF	NDF	Soluble sugars	Starch	PP
Control	90	10	16.8	2.0	22.4	44.4	9.1	1.2	–
ASP	44	56	15.2	<0.5	9.7	24.8	1.5	<0.5	3.4
BNM	49	51	11.7	<0.5	5.2	30.0	3.2	2.1	2.5
EPT	49	51	13.3	<0.5	19.9	40.4	1.3	1.8	3.0
PLC	60	40	17.9	<0.5	8.5	38.2	2.0	1.5	2.6
VDA	72	28	13.8	<0.5	12.7	41.5	2.3	5.7	10.6
ECK	75	25	7.2	<0.5	7.2	26.9	2.0	<0.5	55.3
ULVA	80	20	19.0	<0.5	12.6	30.2	1.6	7.7	2.1

-Indicates not detected

In vitro fermentation

Enteric fermentation is a multistep digestive process where carbohydrates (e.g., hemicellulose, cellulose, and starch) from ingested plant feed are degraded by microbial enzymatic activity into VFAs, mainly acetate, propionate, and butyrate, that serve as the major energy source for ruminants (Kumari *et al.*, 2020). During this process, CO_2 and H_2 are produced as gaseous by-products, and are the substrate for the production of CH_4 by methanogenic archaea (Bhatta & Enishi, 2007; Buddle et al., 2011). Importantly, differences in basal feed substrate composition, the addition of anti-methanogenic supplements, and feed digestibility can influence the total gas production, and proportions of these gases, with flow on effects to other fermentation parameters (e.g., VFAs, OMdeg).

Several studies have demonstrated that the effects of *Asparagopsis* on rumen fermentation are dependent on seaweed quality, based on the concentration of bromoform, and on the composition of ruminant basal feed (Kinley et al., 2020; Maia et al., 2016; Roque et al., 2012, 2021). In this study, inclusion of high-quality *A. armata* at 2% OM resulted in a 16.5% reduction in total gas production compared to the basal feed substrate control (ryegrass hay) with complete inhibition of CH_4 production and a significant increase in H_2 production (Fig 1; Supplementary table S2). This indicates that either a component of the seaweed or the CH_4 inhibition decreased overall fermentation and therefore potentially animal production *in vivo*. When all other seaweeds were included at 2% OM the total gas production was

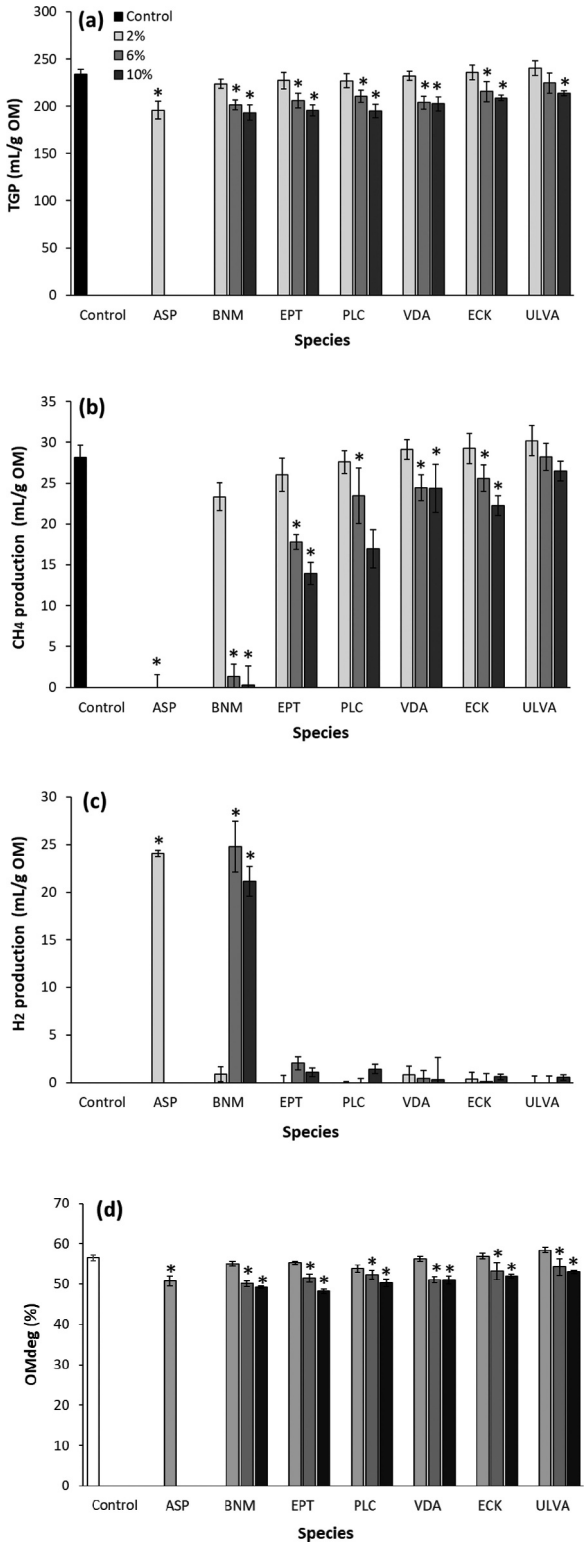

Figure 1. Mean (± SE, n = 3) **(a)** total gas (TGP), **(b)** CH_4, and **(c)** H_2 production (ml g^{-1}), and **(d)** degradability of organic matter (OMdeg, % degraded) at the end of in vitro incubations for freeze-dried perennial ryegrass (RG299, negative control), and seaweed species *Asparagopsis armata* (ASP, positive control), *Bonnemaisonia hamifera* (BNM), *Euptilota formisissima* (EPT), *Plocamium cirrhosum* (PLC), *Vidalia colensoi* (VDA), *Ecklonia radiata* (ECK), and *Ulva* sp. B (ULVA) at inclusion levels of 2%, 6%, and 10% (OM-based) (24 h data).*Indicates treatment significantly different ($p < 0.05$) from control according to PERMANOVA.

unchanged relative to the basal feed substrate control. However, no significant decrease in CH_4 was observed for these treatments. Seaweed inclusions of 6 and 10% OM led to decreases in total gas production of 7.9–17.4%. Inclusion of all seaweeds tested, except *Ulva sp. B*, reduced the production of CH_4 (↓8.9–98.9%) at 6 and 10% OM, with *B. hamifera* reducing the production of CH_4 at 2% OM (Fig 1; Supplementary table S2). Indeed, *B. hamifera* showed the best reduction in CH_4 giving an inclusion level-dependent reduction in the production of CH_4 compared to the basal feed of 17.1% (not statistically significant), 95.4% and 98.9% at inclusion levels of 2%, 6% and 10% OM, respectively. At inclusion levels of 6 and 10% OM an increase in the production of H_2 from 0.9 mg l to 24.8 ml g^{-1} and 21.1 ml g^{-1}, respectively, was observed for *B. hamifera*. At inclusion levels of 6% and 10% OM, *E. formisissima* and *P. cirrhosum* reduced the production of CH_4 by 36.7% and 50.5% (*E. formisissima*), and 16.4% and 39.5% (*P. cirrhosum*), respectively. Both seaweeds led to small increases in the production of H_2 at the highest level of inclusion, consistent with the metabolic hydrogen ([H]) being redirected into alternate biochemical pathways (Janssen, 2010; Leng, 2014; Ungerfeld, 2015, 2018).

A recent meta-analysis of *in vitro* batch and continuous rumen fluid fermentation highlighted that the mean amount of metabolic hydrogen [H] incorporated into H_2 in 100% methanogenesis inhibited fermentations averaged 10% and 6%, respectively (Ungerfeld, 2015). Specifically, the production of one CH_4 molecule from CO_2 requires 8[H] and measured changes in the production of H_2 in methanogenesis inhibited fermentations only accounted for a small proportion of the predicted production of H_2. In this regard, in the current study 100% inhibition of methanogenesis with *A. armata* resulted in only 21% of [H] being redirected into the production of H_2. This is consistent with previous *in vitro* and *in vivo* studies with *Asparagopsis* (Kinley et al., 2020; Machado et al., 2018; Roque et al., 2021; Stefenoni et al., 2021) and other anti-methanogenic products (Hristov et al., 2015; Machado et al., 2016a; Martinez-Fernandez et al., 2016, 2017; Mitsumori et al., 2012). Among the seaweeds tested here, inhibition of methanogenesis by 95.4% and 98.9% by inclusion of *B. hamifera* resulted in a similar proportion of [H] being redirected into the production of H_2 (*i.e.*, ↑23% and 19%) as for the inclusion of *A. armata*. However, the lower rates of inhibition of methanogenesis by 36.7% and 50.5% with the inclusion of *E. formisissima* and *P. cirrhosum*, respectively, resulted in no significant increase in the production of H_2. This result could indicate that other hydrogen utilizing pathways like propionate, valerate, or formate

formation, reductive acetogenesis, or increased microbial biomass production (Leng, 2014; Ungerfeld, 2015) incorporated the excess metabolic [H].

Ingested OM is degraded during ruminal fermentation by an assortment of rumen microbes which generates VFAs, the primary source of energy contributing to the animal's nutrition (Russell et al., 1992). Thus, both OMdeg and VFA production are indicators of fermentation efficiency and reductions in these parameters are generally considered undesirable. In this regard, previous *in vitro* and *in vivo* studies have found that increasing inclusion levels of *Asparagopsis* can lead to a decrease in OMdeg (Machado et al., 2016b, 2018) and VFA production (Li et al., 2016; Machado et al., 2016b, 2018). In the current study, with the exception of *Ulva* sp. B, *OMdeg* was decreased by 8.9% and 4.4–13.5% compared to the basal feed substrate (RG299) with the inclusion of *A. armata* (2% OM) and other seaweeds (≥6% OM), respectively (Fig 1; Supplementary table S2). Compared to basal feed substrate control fermentations, *A. armata* also significantly decreased the production of total VFAs by 22.4%, and *B. hamifera* decreased the production of total VFAs by 20.1 and 25.4% at inclusion levels of 6 and 10% OM, respectively (Table 4). These data are consistent with the decrease in gas production observed for the treatments and is also consistent with earlier work involving different seaweed species (Machado et al., 2014). The molar proportions of butyrate, propionate, and valerate increased for *A. armata* and *B. hamifera* at inclusion levels of 6 and 10% OM, while the proportions of acetate, isobutyrate, and isovalerate decreased (Table 4). *Plocamium cirrhosum* and *E. formisissima* induced similar changes at inclusion levels of 6 and 10% OM, however the effects were less pronounced. Conversely, *V. colensoi, E. radiata*, and *Ulva* sp. B had little or no effect on the production of individual VFAs. The shift from a high acetate: propionate ratio to one which favours the production of propionate is commonly observed with the use of CH_4 inhibitors (Janssen, 2010; Mitsumori et al., 2012), and is due to competition for hydrogen between methanogenesis and propiogenesis pathways (Hungate, 2015; Janssen, 2010; Mitsumori et al., 2012; Moss et al., 2000). This shift in VFA production has important implications on animal production systems with increased propionate production contributing to gluconeogenesis (Aschenbach et al., 2010), while reduced acetate production may lead to a reduction in milk fat percentage (Urrutia et al., 2019). However, the net changes in total VFAs and subsequent theoretical changes in [H] do little to explain the discrepancy between the measured H_2 and the theoretical H_2 production from fermentations with significantly inhibited methanogenesis.

In theory, based on the gross energy of VFAs, diverting the flow of [H] from the production of CH_4 into the production of VFAs and other nutritionally beneficial [H] sinks may increase feed conversion efficiency and

Table 4. Effect of seaweed species *Asparagopsis armata* (ASP, positive control), *Bonnemaisonia hamifera* (BNM), *Euptilota formisissima* (EPT), *Plocamium cirrhosum* (PLC), *Vidalia colensoi* (VDA), *Ecklonia radiata* (ECK), and *Ulva* sp. B (ULVA) (mean ± SE, n = 3) at inclusion levels of 0% (perennial ryegrass, negative control), 2%, 6%, and 10% (OM-based) on total (mmolg^{-1}) and individual volatile fatty acid (VFA) (% total) production at the end of in vitro incubations (48 h data).

Seaweed	Seaweed inclusion (%) OM)	Total VFA [mmol/g]	AC (%)	PR (%)	BU (%)	VA (%)	ISB (%)	ISV (%)
Control	0	6.7 ± 0.1^{B}	66.6 ± 0.4^{B}	19.1 ± 0.4^{B}	10.3 ± 0.1^{B}	1.2 ± 0.1^{B}	1.0 ± 0.0	1.7 ± 0.1
ASP	2	5.2 ± 0.2^{A}	51.4 ± 0.3^{A}	28.1 ± 0.6^{A}	16.3 ± 0.3^{A}	2.0 ± 0.3^{A}	0.7 ± 0.1	1.0 ± 0.1
BNM	2	6.6 ± 0.2^{B}	62.1 ± 1.1^{C}	21.9 ± 1.2^{B}	11.9 ± 0.3^{C}	1.4 ± 0.0	0.9 ± 0.1	1.5 ± 0.2
	6	5.3 ± 0.1^{A}	53.1 ± 0.4^{A}	28.5 ± 0.4^{A}	14.5 ± 0.3^{C}	1.9 ± 0.2	0.7 ± 0.1	1.1 ± 0.0
	10	5.0 ± 0.1^{A}	52.6 ± 0.5^{A}	29.2 ± 0.6^{A}	14.4 ± 0.3^{C}	1.9 ± 0.2	0.7 ± 0.1	1.0 ± 0.1
EPT	2	6.5 ± 0.1^{B}	64.5 ± 0.4^{C}	20.6 ± 0.5^{B}	10.8 ± 0.2^{C}	1.3 ± 0.0	1.0 ± 0.0	1.7 ± 0.1
	6	5.9 ± 0.1^{C}	61.2 ± 0.7^{C}	22.9 ± 0.8^{C}	12.0 ± 0.4^{C}	1.4 ± 0.1	0.9 ± 0.1	1.5 ± 0.2
	10	5.8 ± 0.2^{C}	60.2 ± 0.6^{C}	24.3 ± 0.7^{C}	12.0 ± 0.3^{B}	1.3 ± 0.0	0.8 ± 0.0	1.3 ± 0.1
PLC	2	6.6 ± 0.1^{B}	66.3 ± 0.3^{B}	19.3 ± 0.4^{B}	10.4 ± 0.2^{B}	1.2 ± 0.0	1.0 ± 0.0	1.7 ± 0.1
	6	6.3 ± 0.2^{C}	64.5 ± 0.6^{C}	20.7 ± 0.6^{B}	10.8 ± 0.2^{C}	1.2 ± 0.0	1.0 ± 0.0	1.7 ± 0.1
	10	5.9 ± 0.2^{C}	61.2 ± 1.0^{C}	23.1 ± 1.1^{C}	12.1 ± 0.3^{C}	1.3 ± 0.0	0.8 ± 0.1	1.3 ± 0.2
VDA	2	6.6 ± 0.1^{B}	66.7 ± 0.4^{B}	18.8 ± 0.6^{B}	10.5 ± 0.3^{B}	1.2 ± 0.0	1.0 ± 0.0	1.7 ± 0.1
	6	6.2 ± 0.1^{C}	66.6 ± 0.5^{B}	19.0 ± 0.6^{B}	10.4 ± 0.5^{B}	1.3 ± 0.1	1.0 ± 0.0	1.6 ± 0.1
	10	6.1 ± 0.2^{C}	66.0 ± 0.7^{B}	19.1 ± 0.7^{B}	10.8 ± 0.5^{B}	1.5 ± 0.0	0.9 ± 0.0	1.5 ± 0.1
ECK	2	6.7 ± 0.1^{B}	66.8 ± 0.2^{B}	19.5 ± 0.5^{B}	9.9 ± 0.3^{B}	1.1 ± 0.1	1.0 ± 0.0	1.7 ± 0.1
	6	6.4 ± 0.2^{B}	66.2 ± 0.3^{B}	21.0 ± 0.4^{C}	9.5 ± 0.3^{B}	1.0 ± 0.1	0.9 ± 0.0	1.4 ± 0.1
	10	6.2 ± 0.1^{C}	65.0 ± 1.6^{B}	22.1 ± 1.0^{C}	9.5 ± 0.5^{B}	1.1 ± 0.2	0.8 ± 0.1	1.2 ± 0.2
ULVA	2	6.8 ± 0.1^{B}	66.9 ± 0.3^{B}	19.1 ± 0.5^{B}	10.1 ± 0.2^{B}	1.2 ± 0.0	1.0 ± 0.0	1.7 ± 0.1
	6	6.5 ± 0.1^{B}	66.6 ± 0.3^{B}	19.0 ± 0.6^{B}	10.2 ± 0.3^{B}	1.2 ± 0.0	1.1 ± 0.0	1.8 ± 0.1
	10	6.3 ± 0.1^{C}	66.7 ± 0.4^{B}	19.0 ± 0.6^{B}	10.0 ± 0.3^{B}	1.2 ± 0.0	1.1 ± 0.0	1.9 ± 0.1

Data were analysed separately for each species of seaweed. Treatment is significantly ($p < 0.05$) different from the ^{A}control (0%), ^{B}ASP 2%, or ^{C}both according to PERMANOVA. Acetate (AC), propionate (PR), butyrate (BU), valerate (VA), isobutyrate (ISB), isovalerate (ISV).

the sustainability of ruminant production systems (Johnson & Johnson, 1995); however, the true effect of CH_4 inhibition is yet to be resolved (Ungerfeld, 2018). *In vitro* and *in vivo* studies investigating the effects of the inhibition of methanogenesis on ruminant fermentation frequently find a reduction in the total VFAs (Li et al., 2016; Machado et al., 2016b, 2018); consistent with high partial pressure of H_2 leading to inhibition of microbial dehydrogenases and reduction in fermentation efficiency, feed digestibility, and DM intake (Janssen, 2010; Leng, 2014). Potentially, such a reduction in fermentation efficiency *in vitro* could translate to reductions in animal productivity *in vivo*, however few studies have concurrently measured both total VFAs and animal productivity when methanogenesis has been inhibited (Bayat et al., 2018; Kinley et al., 2020; Melgar et al., 2016), due to challenges in quantifying the constant flux of VFAs in dynamic biological systems. Studies that have, both *in vitro* (Kinley et al., 2016; Machado et al., 2016a, 2016b, Chagas et al., 2019) and *in vivo* (Kinley et al., 2020; Li et al., 2016), show differences in VFA production that depend on the level of inhibition.

While the high partial pressure of hydrogen in the *in vitro* experiments in the current study presumably led to the reduction in total VFAs, these results cannot be used to make predictions on animal production as the partial pressure of hydrogen *in vivo* is much lower due to diffusion and eructation. However, when methanogenesis is inhibited, animal productivity is mixed, with both increased (Haisan et al., 2017; Kinley et al., 2020) and decreased (Roque et al., 2012) productivity demonstrated. For example, inclusion of *A. taxiformis* at 0.1% and 0.2% in the feed of Brahman-Angus cross steers led to reductions in the production of CH_4 of 40% and 98%, with concurrent weight gain improvements of 53% and 42%, respectively (Kinley et al., 2020). Inclusion of *A. armata* at 0.5% OM in the feed of lactating Holstein cows reduced CH_4 production by 26.4% with no change in milk yield or quality measures, but with reduced DM intake, *i.e.*, an increase in feed conversion efficiency (Roque et al., 2012). Higher inclusion levels of *A. armata* (1% OM) resulted in higher CH_4 inhibition (CH_4 production ↓62.7%) but also impacted on animal productivity (milk production ↓11.6%). These examples highlight that *in vitro* fermentations can be used to identify potential CH_4 inhibitors but they are poor predictors of animal productivity responses. In the current study, we identified new seaweed targets worthy of further investigation to identify the precise nature of their active constituents and their activity *in vivo*.

Conclusion

The current study identified several new species of red seaweeds that reduced enteric CH_4 production in a dose-dependent manner. Inclusion of *B. hamifera* into the basal feed substrate (ryegrass) resulted in near elimination of enteric CH_4 production at ≥6% OM inclusion, while *E. formisissima* and *P. cirrhosum* also demonstrated significant anti-methanogenic activity at ≥6% OM inclusion. *A. armata, B. hamifera, E. formisissima* and *P. cirrhosum* had similar effects on measured fermentation parameters to those recorded in previous studies investigating the effects of *Asparagopsis spp*. and other halogenated methane analogues on ruminant fermentation. Importantly, the chemistries of the new seaweed species identified here are unique compared with that of *Asparagopsis* as they do not contain bromoform, the primary bioactive in *Asparagopsis*. These species therefore might provide alternative anti-methanogenic seaweed targets that are less susceptible to the loss of volatile bioactives during processing.

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