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Changing the Proportions of Grass and Grain in Feed Substrate Impacts the Efficacy of *Asparagopsis taxiformis* to Inhibit Methane Production *in Vitro*

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Abstract

Benefits of the red seaweed *Asparagopsis taxiformis* as an ingredient to manage methane (CH_4) emissions from the red meat and dairy industries continue to evolve. *Asparagopsis* has been demonstrated to eliminate enteric CH_4 emissions *in vitro* and reduce it greater than 80% in animals. Variability in animal studies is suspected to be associated with variable inclusion and proportions of grass and grain in the diet. This *in vitro* study aimed to elucidate effects of gradient grass to grain proportions in the fermentation using five steps from 100% Rhodes grass (RG) to 100% barley grain (BG). Gradient inclusion of *Asparagopsis* was in six steps of Control with no inclusion (C), Low (L), Low-Medium (LM), Medium (M), Medium-High (MH), and High (H) levels tested in three fermentation durations (24 h, 48 h, 72 h). There was significant effect of RG/BG and inclusion of *Asparagopsis* such that CH_4 production decreased with increasing *Asparagopsis* independent of RG/BG; however, there was enhanced reduction at greater proportions of BG. Thus, the level of *Asparagopsis* required to completely inhibit CH_4 production *in vitro* was decreased with decreasing RG/BG. Increasing the duration of fermentation had greatest effect on CH_4 at C, L, and LM levels of *Asparagopsis* independent of RG/BG, although magnitude of CH_4 production was greater for higher proportions of BG for the C and L levels. Digestibility of *in vitro* substrate increased with fermentation duration and increasing BG; however, there was no change associated with inclusion levels of *Asparagopsis*. In-

creases in total volatile fatty acids (tVFA) were observed with increased fermentation duration and concomitant with increasing substrate digestion. Increasing proportions of BG induced increase in tVFA. In contrast, and independent of changes in substrate, increasing inclusion of *Asparagopsis* had little effect on tVFA. The acetic and propionic acid ratio (AA:PA) decreased with decreasing RG/BG and increasing *Asparagopsis*. This pattern was most pronounced with 100% BG and MH-H *Asparagopsis* levels. Compared to control, there was decrease in the AA:PA ratio with addition of even L levels of *Asparagopsis* and with L compared to LM inclusion levels. Increasing levels of BG and *Asparagopsis* resulted in significant decreases in AA:PA ratios and CH4 production. This study has confirmed that gradient levels (ratio) of grass and grain in a feed mix impact the antimethanogenic efficacy of *Asparagopsis* during *in vitro* fermentation with rumen fluid.

Keywords

Asparagopsis taxiformis, Enteric Methane, Greenhouse Gas Mitigation, Feed Substrate, Rhodes Grass, Barley Grain, *In Vitro*

1. Introduction

The relevance of antimethanogenic innovations to livestock feeds and feeding systems is evolving under petition from the red meat and dairy industries for methods to manage greenhouse gas (GHG) emissions in effort to meet emissions targets [1]. In Australia, the red meat industry has invested in an ambitious target of reaching carbon neutrality by 2030 (CN30), a program led by Meat and Livestock Australia [2]. The dairy and sheep industries have also shown awareness and willingness to adopt innovative technologies in production systems intended to provide significant GHG emissions reductions.

Diverse innovations with variable impact on enteric methane (CH_4) emissions with variable levels of applicability are being validated, most with specific targets in an industry with a wide range of feeding systems [1] [3] [4]. The challenge for achieving emissions abatement and subsequent speed to impact is intrinsically linked to the feeding system. The challenge increases with decreased influence in the daily behavior of the livestock. For example, the beef feedlot industry is fundamentally well suited to fit-for-purpose total mixed rations (TMR) containing ingredients with a myriad of purposes including CH_4 management. Similarly, but in a reduced capacity, grazing livestock receiving a feed supplement is well suited to receive a formulated daily provision albeit in a pulse format with intake frequency being dependent on the system of delivery. It is common for grazing dairy cattle to receive a supplemental feed at or near milking once or twice per day [5]. Livestock access to supplemental feeds, and voluntary intake of them, varies widely in grazing systems and availability is dependent on the level of nutritional intervention inherent in the system. Extensive systems typical of northern Australia do not typically receive supplemental feeds although they may

have access to lick blocks or pots intermittently. Nutritional intervention in ruminant livestock feeding systems commonly targets increasing intake of efficiently digested and energy dense feed components. In grazing systems, this is commonly as supplemental and/or an improved grassland management. More intensive systems commonly involve provision of higher grain to grass ratio [6]. Nutritional intervention provides a conduit for CH_4 management indirectly through improved feed use efficiency and directly through antimethanogenic feed ingredients.

Reference [7] demonstrated *in vitro* that the tropical legume *Desmanthus* spp. had antimethanogenic potential of up 36% compared to commonly grazed tropical Rhodes grass. That study induced further legume research *in vivo* where provision of increasing Desmanthus in a basal diet of low-quality grass demonstrated a linear increase DMI and reduction of CH_4 [8]. Considering the large numbers of cattle on grasslands in northern Australia a moderate reduction in CH_4 emissions from this sector would make a significant reduction in Australia's total agricultural GHG inventory [8]. Approximately 90% of Australia's beef cattle production occurs on grasslands, however over 40% of Australia's beef is produced on feedlots [9]. The feedlot sector can be rapidly impacted toward reductions in enteric CH_4 emissions using antimethanogenic feed ingredients including the red seaweed Asparagopsis [10] [11] and 3-nitrooxypropanol (3-NOP) [12]. Likewise, these products are effective in mitigation of enteric CH_4 for the dairy industry [13] [14]. The paradox in applicability of antimethanogenic feeds is variable efficacy in inhibition of enteric CH_4 as is demonstrated with variable diets and delivery systems.

Inherent aspects of a feeding system are intrinsic to the magnitude of response to antimethanogenic feeds. This highlights the need for knowledge in how to manage the intake of and inclusion levels of these products relative to their antimethanogenic efficacy in variable feeding systems. Inconsistency in the frequency of intake of a supplement containing the antimethanogenic material is expected to be relevant in management of CH_4 reduction potential. The basal diet itself is expected to be relevant because there is evidence that high concentrate diets produce a more pronounced response to *Asparagopsis* [11]. The latter aspect can be investigated *in vitro* therefore the present study focuses on the efficacy of *Asparagopsis* to inhibit CH_4 production when the basal diet changes with respect to inclusion level and the grass and grain proportions.

Asparagopsis is a potent antimethanogenic feed ingredient for ruminant livestock. The effectiveness has been shown *in vitro* [15] [16] [17] [18] and *in vivo* with variable efficacy [10] [11] [13] [19] [20]. The mode of action is reported to derive from the synthesis of halogenated CH_4 analogues such as bromoform and bromochloroacetic acid [21] [22] that directly inhibit methyl-coenzyme M reductase (MCR) [23] [24] [25] at the final step in the CH_4 formation pathway. Inclusion of *Asparagopsis in vitro* and *in vivo* has resulted in some of the largest CH_4 reductions from a single feed ingredient added to ruminant diets, however response varies between studies. Variability in response to *Asparagopsis* may be

due to a collective of factors including bioactive content of the Asparagopsis product, animal breed, intake of the product relative to basal diet DMI, and basal diet composition.

This study investigates the *in vitro* impact of changing the grass to grain ratio which is a prominent contrast in nutritional intervention between extensive and intensive ruminant livestock production. Published research suggests that increasing the dietary grain to grass ratio increases the antimethanogenic efficacy of *Asparagopsis* [10] [11] which has relative implications for utility of the seaweed for TMR feeding systems and supplemented or non-supplemented grazing systems. The hypothesis is that there is no difference in the minimum effective inclusion level (MEIL) based on antimethanogenic efficacy, or impact on rumen fermentation with inclusion of *Asparagopsis* in a scenario of increasing grain to grass ratio. Specific objectives include: 1) quantification of *in vitro* total CH_4 production (mL/g DMI) as affected by *Asparagopsis* inclusion while increasing the grain to grass ratio; 2) characterization of the of *in vitro* MEIL of a single *Asparagopsis* product required to reduce CH_4 production below the limit of detection as affected by increasing the grain to grass ratio; and 3) characterization of the effect of objectives 1) and 2) on rumen fermentation based on *in vitro* digestibility of substrate dry matter (IVDDM) and *in vitro* volatile fatty acid (VFA) profiles.

2. Materials and Methods

2.1. Preparation of Feed Substrate and Macroalgae

The basal feed substrates used were Rhodes grass (RG; Chloris gayana) locally grown under irrigation and barley grain (BG; Hordeum vulgare) grown in southern Queensland. Both were air-dried and ground to 1 mm, proximate of basal substrates analysis is presented in **Table 1**. Dry matter (DM) was determined by dehydration of substrate at 105˚C until constant weight and organic matter (OM) was determined as loss on combustion at 550˚C for 8 hours [26]. 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.

The *Asparagopsis taxiformis* (hereafter *Asparagopsis*) used in this study was harvested from Humpy Island, Keppel Bay, QLD (23˚3'01"S, 150˚54'01"E) by the Centre for Macroalgal Resources and Biotechnology of James Cook University, Townsville, Queensland AUS. *Asparagopsis* in the gametophyte lifecycle stage was rinsed in clean seawater, placed in mesh bags, and spun for 6 min at 1000 rpm in a commercial washing machine (Fisher and Paykel, Macquarie Park, NSW, AUS) to remove excess water. The fresh biomass was frozen and stored at −20˚C then freeze dried (Forager Food Company, Red Hills, Tas, AUS) to maximise retention of volatile bromoform ($CHBr_3$) [27] as the demonstrated bioactive ingredient [21]. The freeze dried *Asparagopsis* biomass was crushed to 2 - 3 mm (Hobart D340 mixer, Troy, OH, USA) to ensure a uniform product and

Table 1. Nutritional composition of the Rhodes grass and barley grain substrates and *Asparagopsis* biomass (g/kg DM unless stated otherwise).

Composition	Rhodes grass	Barley Grain	*Asparagopsis*
Dry matter (g/kg as fed)	947	871	951
Organic matter	867	980	409
Crude protein	170	93	92
Neutral detergent fiber	648	173	-
Acid detergent fiber	308	45	-
Bromoform (mg/g as fed)	-	-	7.7

stored at −15˚C. Major bioactives concentration (only $CHBr_3$ is reported here) in the *Asparagopsis* was quantified by methanol extraction and subsequent analysis by gas chromatography-mass spectrometry (GCMS) using a modified version of the protocol described by [22].

2.2. Donor Animals and Preparation of Rumen Fluid Inoculum

Rumen fluid inoculum (RF) was collected from four fistulated Brahman (Bos indicus) steers of approximately 430 kg live weight (LW) and fitted with 10 cm Bar Diamond (Parma, OH, USA) rumen cannulas. The steers were maintained at the Commonwealth Scientific and Industrial Research Organization (CSIRO) Lansdown Research Station near Townsville, Qld, AUS (19˚39'27.000"S, 146˚50'04.60"E) according to current guidelines [28] and approved by CSIRO's Qld animal ethics committee on Ethical Clearance Certificate 2018-37. The steers grazed on mixed grasses dominated by RG and were supplemented with irrigated RG ad libitum perpetually. Rumen fluid (RF) was extracted through the cannulas at 7:00 am by sampling from four quadrants of the rumen and hand-squeezing into pre-warmed 1-L stainless steel thermal flasks. The RF was pooled and immediately processed by filtration through a 0.5 mm sieve and combined with an artificial saliva buffer described by [29] buffer (GVB) at a ratio of 1:4 (RF: GVB). Maintenance of 39˚C and mixing of the RF buffer fermentation media (RFB) (Major Science SWB 20 L-3; Saratoga, CA, USA) was continuous to ensure homogeneity. Each fermentation bottle was prepared prior to RF collection with prescribed treatments of RG, BG, and *Asparagopsis* then purged with N2 and capped to maintain anaerobic conditions. Purging was continuous during inoculation and 125 mL of RFB was aspirated into the fermentation bottles using a Dose-It pump (Integra Biosciences, Hudson, NH, USA). The fermentation bottles were then sealed with Ankom RF1 gas production modules (Macedon, NY, USA) and placed in one of six Ratek OM11 incubators (Boronia, Victoria, Australia) maintained at 39˚C and oscillating at 85 RPM.

2.3. *In Vitro* Set up and Experimental Design

The study was a triplicated 5 × 6 × 3 factorial design of five grasses: grain ratios,

six inclusion levels of *Asparagopsis*, and three fermentation durations. The design was developed to compare impact on the efficacy of *Asparagopsis* to reduce CH_4 emissions as affected by gradient inclusion levels of *Asparagopsis* on gradient dietary forage and grain ration formulations using RG and BG, respectively. The factorial consisted of a total of 90 experimental treatment groups. Nutritional composition of RG, BG, and Asparagopsis is reported in Table 1. Five formulation levels of feed substrate [content total 1.0 g OM (≈1.2 g DM)] expressed as a ratio of RG to BG inclusion in the range of; 1) 100% RG [1:0]; 2) 75% RG - 25% BG [3:1]; 3) 50% RG - 50% BG [1:1]; 4) 25% RG - 75% BG [1:3]; and (v) 100% BG [0:1], respectively. *Asparagopsis* inclusion levels included six increasing *Asparagopsis* inclusion levels standardized as mg $CHBr_3$ in 1.0 g OM (≈1.2 g DM) of the feed substrate content (Table 1) in the range of: 1) 0.00 [control]; 2) 0.05 [low, L]; 3) 0.08 [low-medium, LM]; 4) 0.11 [medium, M]; 5) 0.14 [medium-high, MH]; and 6) 0.16 [high, H], respectively. Over the course of five *in vitro* incubation periods all the individual treatment fermentations were incubated (within period) in triplicate to allow for sacrifice of one fermentation bottle at each of three fermentation durations of: 1) 24 h, 2) 48 h, and 3) 72 h, respectively. All treatment groups were monitored in triplicate over the course of the five randomized incubation periods where treatment combinations were different in each period and substrate and *Asparagopsis* treatment groups did not recur in the same incubator to eliminate any chance of incubator or RF batch effect. The data from all sampling periods was combined to provide time series curves representing the effect of RG/BG combinations and gradient *Asparagopsis* inclusion at three fermentation durations over 72.

2.4. Sampling and Analysis

2.4.1. Total Gas and Methane Production

Total gas production (TGP) and CH_4 production were determined using Ankom RF gas production technology (Macedon, NY, USA) with protocols and parameters as described by [15]. Briefly, the Ankom RF modules were set to maximum pressure of 3 psi which when exceeded would vent for 250 milliseconds. Live interval (LI) was set at 60 seconds monitoring of gas production each measurement was corrected for ambient pressure change via ambient Ankom RF monitors. The recording interval (RI) was set to 20 minutes thus LI cumulative pressure change was recorded at each RI as 20 min contributions to the cumulative pressure change over the duration of the fermentation (24, 48, or 72 h). Total cumulative pressure change was converted to TGP using the natural gas law and corrected for absolute volume of individual fermentation bottles.

The TGP was applied in the determination of total CH_4 (mL) expressed as mL/g substrate digested. *In vitro* CH_4 production was determined by analysis of headspace gas relative to TGP while assuming constant homogeneity of bottle headspace. Time series CH_4 production curves were prepared by collection of samples at the three designated timepoint samplings (24, 48, and 72-h). At termination of each incubation period headspace gas samples from individual fer-

mentation bottles were collected through the Ankom RF module vent tube into 10-mL Labco Exetainer vacuum vials (Lampeter, Great Britain). Gas samples were analysed by gas chromatography (GC) on a Shimadzu GC-2014 (Kyoto, Japan) equipped with a Restek (Bellefonte, PA, USA) ShinCarbon ST 100/120 column (2 m × 1 mm × micropacked) with a flame ionisation detector (FID). Column temperature was set to 150˚C, injector at 240˚C, and FID at 380˚C. Ultra high purity N2 was the carrier gas at 25 mL/min and total injection volume was 250 µL.

2.4.2. *In Vitro* Apparent Digestibility of Substrate DM and OM

In vitro digestibility of substrate DM (IVDDM) and OM (IVDOM) was quantified to coincide with CH_4 determinations during 24, 48, and 72-hour fermentation periods as previously described [15]. Immediately following collection of headspace gas samples, the fermentation bottles were chilled to terminate fermentation activity then *in vitro* fluid (IVF) was collected and vacuum filtered through a weighed Duran No. 1 porosity glass fritted crucible containing a 0.5 cm layer of sand filtration aid. The crucible and fermentation residue were oven-dried to constant weight at 105˚C in determination of the remaining substrate DM and subsequent IVDDM determination. The OM in the fermentation residue DM subsequent IVDOM determination was determined as loss on ignition in a muffle furnace at 550˚C for 8 h (Carbolite AAF 11/18; Derbyshire, Great Britain).

2.4.3. Volatile Fatty Acid Production

Volatile fatty production (VFA) for each of the 28, 48, and 72-hour fermentation periods was measured in the IVF as previously described [15]. The sample preparation of IVF for VFA analysis was at a ratio of 4:1 of IVF to 20% metaphosphoric acid spiked to 11 mM with 4-methylvaleric acid (Sigma-Aldrich; Castle Hill, NSW, Australia) as internal standard to achieve a sample concentration of 2.2 mM internal standard. Samples were prepped in 1.5 mL microcentrifuge vials and stored at −20˚C. Sample vials were thawed at 4˚C and centrifuged for 20 min at 13,500 g and 4˚C (Labnet Prism R; Edison, NJ, USA). A 0.5 mL subsamples of clear supernatant was extracted using glass Pasteur pipettes and analysed by GC using a Shimadzu GC-2010 (Kyoto, JPN) equipped with a Restek Stabilwax (30 m × 0.25 mm × 0.25 mm) fused silica column and FID. Initial column temperature was 90˚C and ramped up at 3˚C/min until 155˚C temperature was achieved and was held for 8.3 min. Injector temperature was held at 220˚C and FID at 250˚C. Ultra high purity N2 was used as the carrier gas at 1.5 mL/min and the injection was 1.0 mL.

2.4.4. Statistical Analyses

The effect of gradient RG/BG in the feed substrate (1:0, 3:1, 1:1, 1:3, 0:1) and gradient *Asparagopsis* inclusion (0, L, LM, M, MH, H) at three fermentation durations (24 h, 48 h, 72 h) was analysed for CH_4 and VFA production, IVDDM, and IVDOM in a 5 × 6 × 3 factorial experiment as a univariate repeated-measures

ANOVA using the General Linear Model procedure of SPSS Statistics 27 (IBM Corp, Armonk, NY). Differences among means were tested by One Way ANOVA with LSD Post Hoc Multiple Comparisons procedure of SPSS for significant differences between the substrate treatments, *Asparagopsis* treatments. Differences in treatments were tested by pairwise comparisons (LSD test). Effects were declared significant at $P < 0.05$ and $P = 0.05 - 0.10$ were considered as a trend.

3. Results

3.1. Methane Production

Inhibition of CH_4 production during simulated rumen fermentations *in vitro* as affected by gradient inclusion levels of *Asparagopsis* and RG/BG in the substrate is displayed as reduction response over 24 (a), 48 (b), and 72-hour (c) incubation periods (Figure 1). The CH_4 production decreased with increasing inclusion level of *Asparagopsis* independent of RG/BG ratio ($P < 0.001$) and the efficacy of reduction was further enhanced by greater proportions of BG. Thus, the level of *Asparagopsis* required to completely inhibit CH_4 production was decreased concomitant with decreasing RG/BG ratio as presented in Figure 1. Therefore, it was demonstrated that increasing the dietary grain content relative to grass increases the antimethanogenic response to *Asparagopsis* during *in vitro* rumen

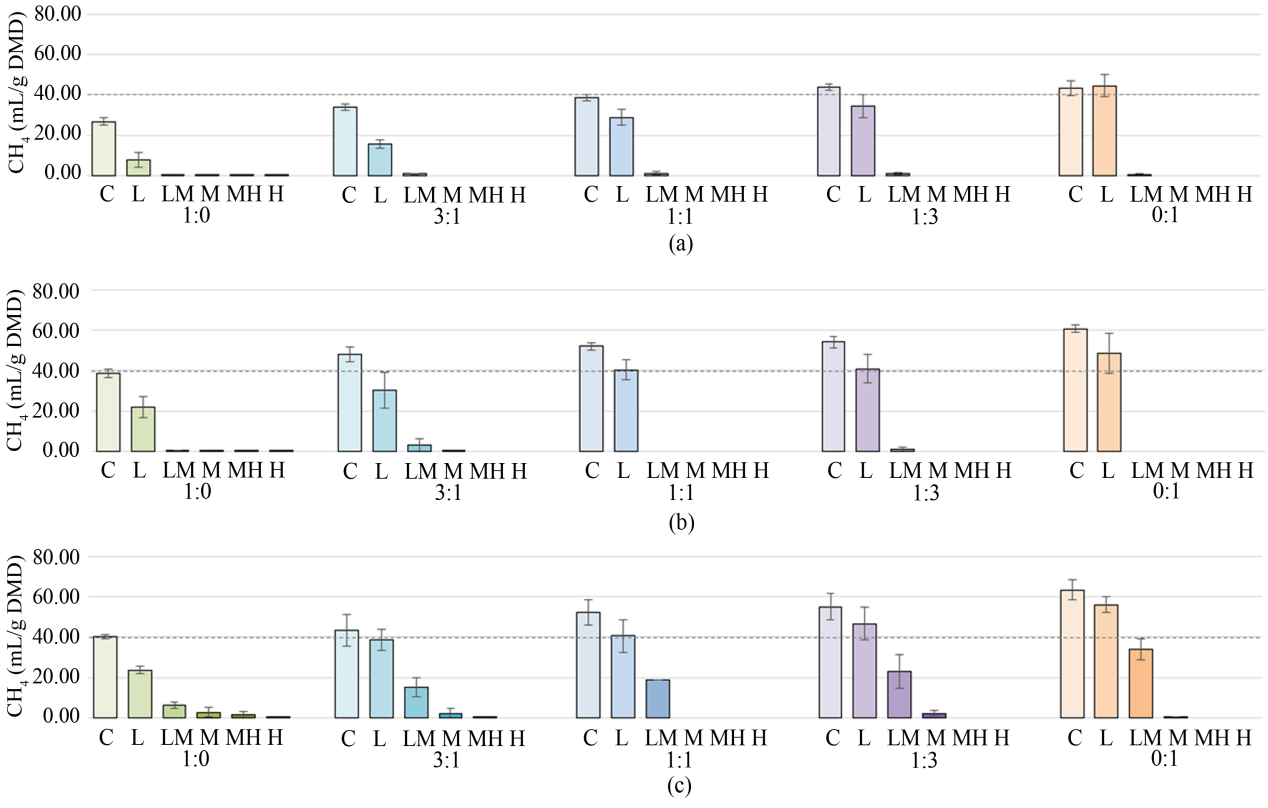

Figure 1. Effect of inclusion levels of *Asparagopsis* (C, L, LM, M, MH, H) on methane production (mL/g DMD) at different Rhodes grass to barley grain ratios (1:0, 3:1, 1:1, 1:3, 0:1) after 24 h (a), 48 h (b) and 72 h (c) *in vitro* fermentation. Data points are treatment means and error bars represent standard errors.

fermentation. Compared to the Control, the L inclusion level of *Asparagopsis* in 1:0 RG/BG (100% RG) resulted in significant CH4 reduction of 70% ($P < 0.001$) in the first 24 h of fermentation. Antimethanogenic efficacy in 100% RG was reduced over time but remained significant with CH_4 inhibition of 43% ($P < 0.001$) and 41% ($P < 0.001$) after 48 h and 72 h of fermentation, respectively. Increasing the level of *Asparagopsis* increased the efficacy and consistency of CH_4 inhibition and the LM inclusion level of *Asparagopsis* in 100% RG induced 99% CH_4 reduction for up 48 h and reducing in efficacy to 84% reduction approaching 72 hours. The M and MH, levels of *Asparagopsis* inclusion in 100% RG eliminated CH_4 production for up to 48 h then marginally reduced in efficacy to 93% and 96% reduction approaching 72 hours, respectively. In contrast when the substrate was 0:1 RG/BG (100% BG), the response to *Asparagopsis* at the L inclusion level was marginal at 0%, 20%, and 12% for the 24 h, 48 h, and 72 h fermentation durations respectively. However, with each gradient step in *Asparagopsis* level the response magnified such that the LM level induced 99%, 100%, and 45% reductions over 24 h, 48 h, and 72 h, respectively. The M level represented the inclusion level where elimination of CH_4 was virtually sustained over time. The H level of inclusion eliminated CH_4 production in all the fermentations independent of RG/BG.

The contrasting antimethanogenic response to increasing *Asparagopsis* inclusion when using fermentation substrates of 100% RG and 100% BG was further elucidated by examination of the gradient effect with reduction in the proportion of RG and increase in BG. The RG/BG ratio was a influencing factor in efficacy of reduction of CH_4 with increasing BG by inducing more sensitivity to *Asparagopsis* (**Figure 1**). The response to RG/BG at 3:1 (25% BG) of diet substrate was similar 1:0 (100% RG) and sustained elimination of CH_4 was only achieved at the H level of *Asparagopsis* inclusion. The 1:1 RG/BG ratio (50% RG, 50% BG) demonstrated similar CH_4 reductions as the 1:3 (75% BG) with 21%, 24%, and 15% for the L inclusion level and 97%, 98%, and 58% for the LM level over 24 h, 48 h, and 72 h, respectively. With RG/BG at 1:1 and 1:3 a sustained elimination of CH_4 was achieved at the M *Asparagopsis* inclusion level signaling a shift to improved antimethanogenic response.

3.2. Dry Matter Digestibility

In vitro digestion of dry matter (IVDDM) is shown in **Table 2** for fermentations over 24 h, 48 h, and 72 h incubation periods. Significant increases in IVDDM were demonstrated at all fermentation durations with decreasing RG and increasing of BG proportions in the diet substrate over time ($P < 0.001$). This is a demonstration of the lower inherent RG-IVDDM of 63% 72%, and 74% compared to BG-IVDDM of 83%, 90%, and 91% at 24 h, 48 h, and 72 h, respectively. This is typical of rumen *in vitro* batch culture fermentations such as Ankom where the IVDDM and IVDOM rates of digestion peaks before 36 h and slows down such that it is approaching completion by 48 h independent of the diet

Table 2. Effect of inclusion levels of *Asparagopsis* (C, L, LM, M, MH, H) on dry matter digestibility at different Rhodes grass (RG) to barley grain (BG) ratios (1:0, 3:1, 1:1, 1:3, 0:1) and incubation periods (24 h, 48 h, 72 h). Means with different letters within a column differ significantly at $P < 0.05$.

Diet (RG:BG)	*Asparagopsis* inclusion level	Incubation period, h					
		24		48		72	
		Mean	SE	Mean	SE	Mean	SE
1:0	C	0.629^{A}	0.019	0.716^{AB}	0.009	0.736^{A}	0.022
	L	0.627^{A}	0.013	0.726^{B}	0.005	0.747^{A}	0.020
	LM	0.615^{A}	0.013	0.696^{AB}	0.007	0.738^{A}	0.018
	M	0.640^{A}	0.024	0.691^{AB}	0.015	0.726^{A}	0.021
	MH	0.637^{A}	0.013	0.705^{AB}	0.016	0.730^{A}	0.011
	H	0.641^{A}	0.014	0.690^{A}	0.013	0.725^{A}	0.017
	P-value	0.882		0.232		0.956	
3:1	C	0.669^{A}	0.017	0.751^{A}	0.023	0.790^{A}	0.013
	L	0.700^{A}	0.004	0.763^{A}	0.010	0.773^{A}	0.020
	LM	0.689^{A}	0.007	0.758^{A}	0.013	0.781^{A}	0.026
	M	0.684^{A}	0.017	0.760^{A}	0.007	0.783^{A}	0.026
	MH	0.682^{A}	0.007	0.754^{A}	0.007	0.752^{A}	0.027
	H	0.666^{A}	0.007	0.760^{A}	0.012	0.762^{A}	0.016
	P-value	0.319		0.988		0.814	
1:1	C	0.715^{A}	0.017	0.800^{A}	0.012	0.831^{A}	0.009
	L	0.721^{A}	0.021	0.794^{A}	0.015	0.830^{A}	0.013
	LM	0.714^{A}	0.027	0.800^{A}	0.011	0.839^{A}	0.003
	M	0.716^{A}	0.008	0.803^{A}	0.006	0.830^{A}	0.012
	MH	0.709^{A}	0.017	0.808^{A}	0.007	0.818^{A}	0.006
	H	0.696^{A}	0.019	0.788^{A}	0.006	0.827^{A}	0.011
	P-value	0.955		0..803		0.832	
1:3	C	0.764^{A}	0.010	0.855^{A}	0.004	0.867^{A}	0.004
	L	0.768^{A}	0.007	0.849^{AB}	0.011	0.871^{A}	0.006
	LM	0.766^{A}	0.004	0.846^{AB}	0.010	0.876^{A}	0.015
	M	0.763^{A}	0.008	0.830^{B}	0.007	0.866^{A}	0.008
	MH	0.761^{A}	0.015	0.833^{AB}	0.005	0.854^{A}	0.010
	H	0.766^{A}	0.002	0.832^{AB}	0.007	0.852^{A}	0.014
	P-value	0.994		0.183		0.439	

Continued

0:1	C	0.833^{B}	0.009	0.896^{A}	0.004	0.908^{A}	0.007
	L	0.824^{AB}	0.010	0.890^{A}	0.004	0.929^{B}	0.004
	LM	0.806^{AB}	0.007	0.891^{A}	0.012	0.927^{BC}	0.007
	M	0.792^{A}	0.0188	0.869^{A}	0.016	0.907^{A}	0.005
	MH	0.821^{AB}	0.010	0.869^{A}	0.008	0.910^{AC}	0.003
	H	0.794^{A}	0.008	0.868^{A}	0.010	0.906^{A}	0.009
	P-value	0.095		0.201		0.038	

[15]. However, independent of the IVDDM changes induced by gradient RG/BG, there was no differences in IVDDM induced by increasing inclusion of *Asparagopsis* after 24 h, 48 h, or 72 h with P-values of P = 0.626, P = 0.084, and P = 0.145, respectively.

3.3. Volatile Fatty Acid Production

Total volatile fatty acids (tVFA) including the major subspecies acetic acid (AA), propionic acid (PA), and butyric acid (BA) are captured in the *in vitro* fluid in the fermentation bottles during *in vitro* batch culture fermentation and accumulate for the specified duration of the experimentation. For simplicity, Table 3 presents the impact of RG/BG and *Asparagopsis* on VFA production at the maximum accumulation point in this study which is response over 72 h of fermentation. However, VFA production during fermentations of 24 h and 48 h are provided as supplement materials (Table S1 and Table S2, respectively).

In addition to the expected increases in tVFA (mM) that typically occur as result of increased time and subsequent increasing IVDDM, for all levels of *Asparagopsis* including control, the gradient change in dietary composition had significant effect. Increasing proportions of BG in the diet substrate induced increase in tVFA during 72 h of *in vitro* fermentation (P < 0.001). The 100% RG diet substrate produced the least tVFA, then a plateau was observed for mixed rations, and the highest tVFA production occurred for the 100% BG substrate. In contrast, and independent of changes in substrate, increasing levels of *Asparagopsis* had little effect on production of tVFA. Supplementary Table S1, Table S2 and Table 3 show that there was no difference in tVFA within the fermentation durations as confirmed with the minimum P-values for all the combined RG/BG ratios of P = 0.352, P = 0.247, and P = 0.246 for the 24 h, 48 h, and 72 h fermentations, respectively.

Table 3 shows the major subspecies VFA's as relative proportions (%) of tVFA produced during 72 h of fermentation. Acetic acid is consistently produced in the highest proportion independent of the diet compositions (P < 0.001) and levels of *Asparagopsis* inclusion (P < 0.001). However, although AA remains proportionately the highest of tVFA, increasing levels of BG and *Asparagopsis* both decrease AA independently (P < 0.001) such that at the highest of

Table 3. Effect of inclusion levels of Asparagopsis (C, L, LM, M, MH, H) on volatile fatty acid profile at different Rhodes grass (RG) to barley grain (BG) ratios (1:0, 3:1, 1:1, 1:3, 0:1) after 72 h *in vitro* fermentation. Means with different letters within a column differ significantly at $P < 0.05$.

Diet (RG:BG)	*Asparagopsis* inclusion level	Total VFA (mM)		AA (%)		PA (%)		BA (%)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
1:0	C	55.50^{A}	5.07	71.03^{D}	0.82	19.09^{A}	0.67	5.03^{A}	0.20
	L	49.60^{A}	5.93	57.86^{C}	1.45	25.67^{B}	1.24	9.17^{B}	0.60
	LM	46.93^{A}	3.34	51.99^{B}	0.80	28.52^{BC}	0.42	11.66^{C}	0.67
	M	43.16^{A}	2.28	50.23^{AB}	1.63	29.57^{C}	0.85	11.80^{C}	1.00
	MH	45.32^{A}	4.49	49.93^{AB}	1.40	30.66^{CD}	1.07	12.00^{C}	0.84
	H	43.00^{A}	4.34	48.16^{A}	1.33	33.23^{D}	1.43	12.79^{C}	0.81
	P-value	0.442		<0.001		<0.001		<0.001	
3:1	C	65.57^{A}	5.15	68.22^{D}	0.06	18.34^{A}	0.58	8.56^{A}	0.34
	L	63.43^{AB}	5.71	58.76^{C}	3.26	22.49^{B}	1.57	11.67^{B}	1.17
	LM	57.46^{AB}	5.16	49.44^{B}	1.37	27.57^{C}	0.33	15.04^{C}	1.00
	M	55.70^{AB}	3.33	46.31^{AB}	0.95	29.02^{CD}	0.51	16.69^{CD}	1.04
	MH	54.15^{AB}	3.17	45.35^{AB}	1.25	31.70^{D}	0.98	17.11^{CD}	0.89
	H	51.47^{B}	2.96	43.43^{A}	1.16	34.70^{E}	0.96	17.86^{D}	0.72
	P-value	0.246		<0.001		<0.001		<0.001	
1:1	C	66.83^{A}	7.39	65.51^{C}	0.63	19.20^{A}	0.65	10.47^{A}	0.16
	L	63.35^{A}	7.30	52.82^{B}	4.06	24.76^{AB}	2.51	14.88^{B}	1.22
	LM	61.06^{A}	7.69	44.32^{A}	1.06	28.47^{B}	1.10	18.77^{C}	0.24
	M	61.10^{A}	7.89	43.77^{A}	1.22	29.74^{BC}	1.42	19.21^{CD}	0.31
	MH	52.91^{A}	7.70	21.20^{A}	1.88	34.88^{CD}	2.32	20.10^{CD}	0.48
	H	50.85^{A}	7.96	39.07^{A}	1.54	37.17^{D}	2.15	20.89^{D}	0.27
	P-value	0.665		<0.001		<0.001		<0.001	
1:3	C	71.88^{A}	5.70	61.50^{D}	0.54	21.71^{A}	0.77	11.67^{A}	0.51
	L	68.31^{A}	6.85	53.41^{C}	3.97	24.82^{A}	1.82	14.93^{B}	1.10
	LM	64.76^{A}	4.70	43.24^{B}	0.73	29.34^{B}	0.16	19.33^{C}	1.12
	M	62.72^{A}	5.41	40.89^{AB}	0.82	32.07^{BC}	0.83	20.81^{CD}	0.67
	MH	60.31^{A}	6.68	38.44^{AB}	1.25	35.21^{CD}	1.85	22.25^{D}	0.07
	H	56.13^{A}	5.47	35.68^{A}	2.23	37.52^{D}	1.87	23.25^{D}	0.86
	P-value	0.497		<0.001		<0.001		<0.001	

Continued

0:1	C	73.61^{A}	6.25	58.02^{C}	0.73	23.56^{A}	0.92	13.43^{A}	0.24
	L	71.65^{A}	8.46	52.82^{C}	2.54	26.36^{AB}	1.52	14.97^{A}	1.15
	LM	61.96^{A}	12.1	39.70^{B}	1.07	33.62^{BC}	2.50	21.15^{B}	0.57
	M	59.55^{A}	13.1	35.23^{AB}	3.34	37.87^{C}	3.88	23.02^{BC}	0.42
	MH	55.82^{A}	9.04	29.65^{A}	3.16	40.49^{C}	2.54	26.37^{C}	1.35
	H	54.83^{A}	7.63	30.37^{A}	1.52	41.15^{C}	2.53	25.31^{C}	0.67
	P-value	0.652		<0.001		0.001		<0.001	

levels of both (0:1 and MH-H) the PA were marginally higher than AA. The effect of *Asparagopsis* on AA was more pronounced with the higher inherent AA when RG/BG was high (1:0 and 3:1) demonstrated by larger drops in AA proportion. This effect is evident but less pronounced with increasing BG and the pattern is more stable as the AA proportion in the Control decreases. As the proportion of BG increases in the substrate, *Asparagopsis* seems to have a larger effect on reduction of AA between L and LM inclusion rates. In contrast to AA, the PA proportion increased in a stepwise manner following both decreasing RG/BG gradient levels ($P < 0.001$) and increasing *Asparagopsis* inclusion ($P < 0.001$). The largest increases in PA were demonstrated to occur with the combination of 100% BG (0:1) and highest level of *Asparagopsis* (H). The production of BA was similar compared to PA and increased with decreasing RG/BG ($P < 0.001$) and increased with increasing *Asparagopsis* inclusion ($P < 0.001$).

Figure 2 illustrates that the ratio of AA:PA decreases with decreasing RG/BG ($P < 0.001$) and increasing *Asparagopsis* inclusion ($P < 0.001$). The pattern was most pronounced with 100% BG and MH-H *Asparagopsis* inclusion levels. Compared to control there was significant drops in the AA:PA ratio with addition of even L levels of *Asparagopsis* ($P < 0.001$ - 0.05) and with L compared to LM inclusion levels ($P < 0.001$ - 0.05). Comparatively, the between level decrease in AA:PA was diminished with inclusion levels M-H but the decreasing AA:PA pattern remained.

4. Discussion

4.1. Methane Production

This study demonstrated a large degree of contrast in CH_4 production using *in vitro* fermentation compared to *in vivo* animal studies. A notable observation of the current study is the increase in CH_4 emissions when BG is added in gradient levels to the fermentation substrate. This result is contrary to results found when measuring CH_4 emissions from animals [30] [31], however does align with other *in vitro* studies. For example, three independent *in vitro* studies found elevated CH_4 production in high starch feeds when compared to high fiber feeds [32] [33] [34]. All three studies concur that this is likely due to the increase in rapidly fermentable carbohydrates compared to the more structural carbohydrates

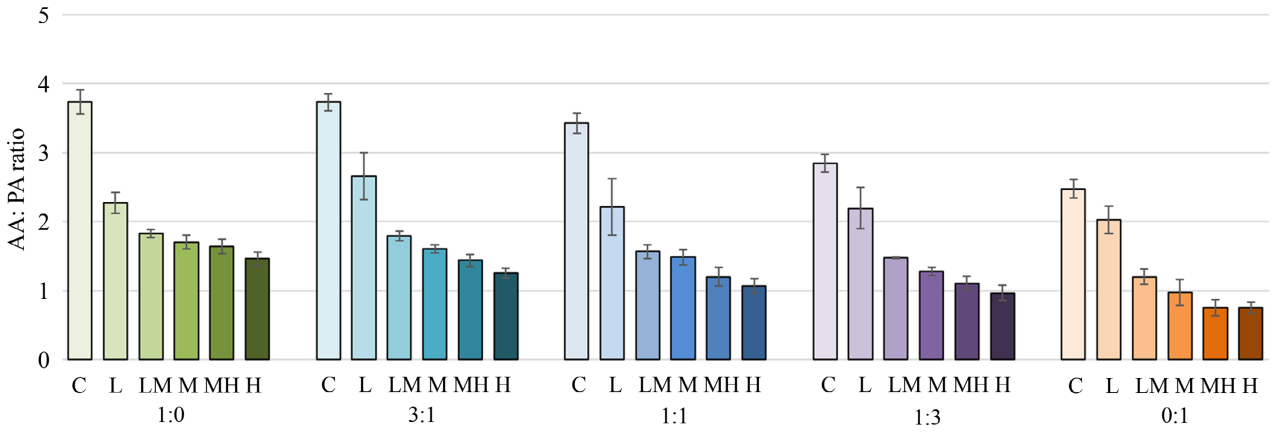

Figure 2. Effect of inclusion levels of *Asparagopsis* (C, L, LM, M, MH, H) on acetic acid: propionic acid ratio (AA:PA) at different Rhodes grass (RG) to barley grain (BG) ratios (1:0, 3:1, 1:1, 1:3, 0:1) after 72 h *in vitro* fermentation. Data points are treatment means and error bars represent standard errors. Effects of inclusion levels were significant at $P < 0.001$ independent of RG/BG.

found in high fiber feeds which typically take longer for microbial populations to ferment. Another explanation for the increase in CH_4 with highly digestible feeds is due to the symbiotic nature of rumen ciliates, which are prolific fermenters of readily fermentable carbohydrates, and methanogens through hydrogen transfer [35] and the fact that *in vitro* cultures cannot simulate variable rumen retention rates for feeds of variable digestibility. Reference [34] also reported that with rapid fermentation of starchy feeds there is typically a concomitant drop in rumen pH levels which are protected against pH drop in a closed system during buffered *in vitro* batch culture. A significant drop in ruminal pH can inhibit fibrolytic bacteria *in vivo* thus reducing digestion and subsequently carbon dioxide (CO_2) and hydrogen (H_2) available for methanogenesis. However, this scenario is unlikely in the strongly buffered *in vitro* batch cultures lending to higher CH_4 production per kg DM from higher levels of BG *in vitro* compared to *in vivo*.

Even with increases in CH_4 emissions with high grain diets *in vitro* the MEIL of *Asparagopsis* required for sustained CH_4 mitigation is consistent with previous work done *in vivo*. Reference [11] tested two inclusion levels of *Asparagopsis* compared to a control group using growing Angus-Hereford steers. In three diet formulation phases of starter-transition-finishing diet of 63 d, 21 d, and 63 d, respectively. In their study with the diet phases of high, mid and low forage inclusion the researchers found that as forage (alfalfa and wheat hay + distillers grain) content decreased, and grain (rolled corn) increased, the efficacy of antimethanogenesis increased concurrently. This is consistent with the current study and *Asparagopsis* was demonstrated to have increased antimethanogenic efficacy with higher BG as RG/BG was decreased. This was also demonstrated with other CH_4 reducing feed additives, and 3-NOP was reported as having a linear relationship between MCR and dietary NDF concentrations in the rumen, thus an increase in efficacy of *Asparagopsis* to inhibit MCR enzymes when NDF concentrations are low may be observed [12].

The implications of variable antimethanogenic response to *Asparagopsis* and

how it would manifest in practice is of interest to the livestock industry. The feeding systems, and hence diet formulations, can vary widely and include but limited to, livestock type, breed, format of feeding, and feed formulation, which are features impacting the CH_4 emissions profile for those systems. Feedlot beef and sheep systems, as the name suggests, receive formulated rations designed to drive high productivity through increasing feed conversion efficiency and the diet is typically high in energy dense component, namely grain. This provides for an opportune system predisposed to high levels of relative CH_4 mitigation with a low MEIL of *Asparagopsis*. This *in vitro* study confirms the indication derived from collective, but less diet directed, *in vivo* studies [10] [11], thus more grain reflects less *Asparagopsis* required for high level CH_4 mitigation. Feedlot systems are inherently the "low hanging fruit" for ease of delivery of *Asparagopsis* and efficacy of antimethanogenic response. However, grazing systems make up much larger contingents to cattle and sheep in Australia and for GHG emissions inventory management it is important to mitigate CH_4 emissions in grazing systems. Evolution of *Asparagopsis* delivery into supplemented feeding systems would be the logical subsequent step. Supplemented systems are largely grass diets of variable digestibility and knowledge derived from this study will assist in planning the delivery system and loading of *Asparagopsis* in supplements. Dairy systems generally receive good quality forage and commonly offer a supplement at, or near, time of milking once or twice per day. Knowledge of a slightly higher MEIL that may be partially impacted by forage quality and grain supplementation will drive further investigation into supplement formulation and palatability management. Currently there is no technology for consistent delivery of feed supplements in large scale beef and sheep grazing systems in Australia. These extensive systems have low level of animal and producer interaction thus delivery and confirmation of intake is problematic. Further confounding efficacy of antimethanogenesis is seasonal changes in forage availability and quality. To have important and sustained GHG emissions reduction realized in extensive systems requires significant evolution in knowledge and technology which would be profitable to the sector. This study has confirmed that all feeding systems can realize large CH_4 emissions reduction but the strategies to achieving it varies just as widely as the livestock industry.

4.2. Digestibility

Significant increases in IVDDM were observed with increasing levels of BG compared to RG in the fermentation substrate composition. As described in **Table 1** this is the result of proportionally higher levels of readily digestible carbohydrates found in BG compared to RG which is a more fibrous substrate [34]. Beneficially, from the perspective of using *Asparagopsis* in ruminant livestock feed, the seaweed did not induce significant changes in digestibility at any of the *Asparagopsis* inclusion rates. This is consistent with other *in vitro* studies that reported little effect on IVDOM when *Asparagopsis* was included at levels up to 10% OM [16] [17]. Reference [15] further examined the effect of *in vitro* inclu-

sion levels of *Asparagopsis* with a gradient series of inclusion from 0% to 10% OM. The study demonstrated there was no impact on IVDOM at inclusions level up to 5%, however approaching levels of 10% there was reduction in IVDOM. The highest level of inclusion (H) in the present study was equivalent to 1.2% OM, comparatively *in vivo* studies feeding *Asparagopsis* have demonstrated antimethanogenic efficacy at MEIL as low as 0.2% OM [10] and 0.5% [11]. Our findings are consistent with other studies and contribute to the growing body of evidence demonstrating that digestibility is not impacted by the inclusion of Asparagopsis and that it is independent of the grass and grain composition. Clearly, there is an extensive margin of consistency for digestibility when feeding *Asparagopsis* at the MEIL.

4.3. Volatile Fatty Acid Production

Microbial fermentation of feed in the rumen accounts for approximately 50% - 70% of the animal's available dietary energy and is largely in the form of VFAs [36]. The VFA profile is dominated by AA, PA, and BA which are the most abundant VFA's produced in the rumen [37]. This study is consistent with previous *in vitro* research that has reported little to no changes in total VFA production induced by *Asparagopsis* inclusion [15] [18]. The same observation was also reported in *Asparagopsis in vivo* studies [10] [19]. Changes in tVFA production were induced by substrate RG/BG changes, with the lowest tVFA observed in high RG diets with gradual increases as dietary BG increased. As with CH_4 production *in vitro* the increased tVFA may be associated to inherently greater digestibility of BG diets compared to RG diets as well as strongly buffered pH within the fermentation bottles. Due to increased production of VFAs in the rumen it is typical for a drop in pH due to the acidic nature of VFAs. This causes negative feedback for microbes because VFA absorption rate is slower than the rate of production in the rumen [38] [39]. The relative abundance of VFA species in the profile is largely reliant on the types of feed substrates being fermented. Dominant with grass based diets, AA is mainly liberated by bacterial and fungal populations responsible for the degradation of fibrous and structural carbohydrates. In contrast PA and BA are typically produced by bacterial and ciliate protozoal populations responsible for the degradation of starchy, more soluble carbohydrates [40] [41].

Concomitant with VFA during feed substrate degradation CO_2 and H_2 are also produced and the animal is not able to utilize these directly and may be detrimental when accumulating in the rumen [38] [39]. Methanogens can take advantage of these excess end products in their own metabolism which results in feed energy waste as CH_4 which is an inefficiency of rumen digestion. Increased H_2 pressure in the rumen is of interest when CH_4 production is dramatically reduced as is possible with *Asparagopsis* inclusion in the diet. Pathways that utilize H_2, and offer H_2 sinks, including VFA production but more specifically the longer chain species of PA ($C_3H_6O_2$) and BA ($C_4H_8O_2$) compared to AA ($C_2H_4O_2$) [42]. This study demonstrated that increasing *Asparagopsis* inclusion has a posi-

tive impact on increasing the proportion of PA and BA in the tVFA profile indicating that these pathways are being upregulated to utilize excess H_2 present because of CH_4 inhibition. Furthermore, AA/PA ratios decreased with the increase of *Asparagopsis* inclusion rates which has been demonstrated both *in vitro* [15] [18] and *in vivo* [10] [19] and may suggest increased energy availability to the animal because PA is the main glucose precursor in ruminants [43].

5. Conclusion

This study has confirmed that gradient levels (ratio) of grass and grain in a feed mix impact the antimethanogenic efficacy of *Asparagopsis* during *in vitro* fermentation with rumen fluid. It was demonstrated that feed formulations with higher levels of grass respond to *Asparagopsis* less effectively than formulations with higher levels of grain. Therefore, the MEIL is higher for diets high in grass compared to diets high in grain. Subsequently, less *Asparagopsis* is required to reduce CH_4 to below detection with *in vitro* substrates typical of feedlot diets compared to grazing systems. This study provides evidence and implications for the livestock industry in that feedlot style diets are particularly well suited to *Asparagopsis* for dramatic reduction in GHG emissions. However, the evidence suggests grazing systems will respond well to *Asparagopsis* but technology for stabilization and delivery aimed at consistent intake of the appropriate MEIL is deficient. Changes in IVDDM were due to fermentation duration and change in grass and grain components in the substrate, such that increasing the fermentation duration and BG component both resulted in greater IVDDM. *Asparagopsis* inclusion did not induce changes in IVDDM independent of RG/BG, and IVDDM was approaching completion after 48 h of fermentation. Likewise, tVFA production was increased by increasing BG and fermentation time, concomitant with increase in IVDDM. Notably, *Asparagopsis* did not induce changes in tVFA. The VFA species were impacted by RG/BG such that AA and PA proportions of tVFA were inversely related such that high RG produced the most AA, while high BG produced the most PA, and BA followed the same pattern as PA but at lower proportions of tVFA. Increasing *Asparagopsis* inclusion decreased the tVFA proportion of AA, and increased the proportion of PA, and BA which was concomitant with decrease in CH_4 production. Therefore, AA:PA decreased with increase in BG and *Asparagopsis*, and with decrease in CH_4 production.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplementary

Table S1. Effect of inclusion levels of *Asparagopsis* (C, L, LM, M, MH, H) on volatile fatty acid profile at different Rhodes grass (RG) to barley grain (BG) ratios (1:0, 3:1, 1:1, 1:3, 0:1) after 24 h *in vitro* fermentation. Means with different letters within a column differ significantly at $P < 0.05$.

Diet (RG:BG)	*Asparagopsis* inclusion level	Total VFA (mM)		AA (%)		PA (%)		BA (%)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
1:0	C	41.55^{A}	3.84	75.14^{C}	0.63	18.90^{A}	0.49	3.78^{A}	0.40
	L	33.47^{A}	4.99	61.56^{B}	2.72	27.60^{B}	1.09	8.11^{B}	0.35
	LM	34.12^{A}	2.29	57.89^{AB}	0.41	30.59^{C}	0.88	9.33^{BC}	0.39
	M	33.93^{A}	1.73	57.58^{AB}	1.91	31.28^{C}	1.04	9.40^{BC}	0.85
	MH	32.83^{A}	1.37	56.59^{A}	0.89	31.83^{C}	0.93	9.51^{BC}	0.52
	H	33.35^{A}	1.79	56.32^{A}	0.84	32.17^{C}	0.91	9.80^{C}	0.32
	P-value	0.352		<0.001		<0.001		<0.001	
3:1	C	48.99^{A}	4.27	70.72^{C}	0.95	19.16^{A}	0.59	7.81^{A}	0.21
	L	44.70^{A}	5.76	59.61^{B}	3.52	24.57^{B}	2.40	12.33^{B}	1.18
	LM	38.82^{A}	3.66	50.19^{A}	0.91	32.71^{C}	0.47	15.36^{C}	0.73
	M	39.49^{A}	3.56	47.87^{A}	0.57	34.20^{C}	0.43	15.81^{C}	0.85
	MH	40.44^{A}	4.06	47.55^{A}	0.92	34.01^{C}	0.22	16.47^{C}	0.92
	H	36.39^{A}	3.35	47.84^{A}	0.72	34.54^{C}	0.75	15.70^{C}	1.04
	P-value	0.369		<0.001		<0.001		<0.001	
1:1	C	48.56^{A}	4.82	66.64^{C}	0.51	20.50^{A}	0.11	10.52^{A}	0.52
	L	43.92^{AB}	5.51	51.79^{B}	2.64	28.33^{B}	1.81	16.33^{B}	0.49
	LM	40.66^{AB}	1.58	44.17^{A}	0.60	34.11^{C}	0.95	19.69^{C}	1.01
	M	38.85^{AB}	2.86	43.65^{A}	1.64	35.46^{C}	0.72	19.15^{BC}	1.08
	MH	35.69^{B}	4.81	41.43^{A}	0.99	37.36^{C}	0.54	19.46^{C}	1.18
	H	43.55^{AB}	1.81	43.98^{A}	1.24	35.05^{C}	2.13	19.19^{BC}	1.13
	P-value	0.355		<0.001		<0.001		<0.001	
1:3	C	49.21^{A}	5.12	61.23^{C}	0.67	24.19^{A}	0.49	12.25^{B}	0.60
	L	47.03^{A}	6.08	48.58^{B}	4.55	30.52^{B}	2.94	18.30^{B}	2.23
	LM	40.95^{A}	3.34	38.59^{A}	0.51	38.14^{C}	0.54	21.38^{BC}	1.52
	M	41.77^{A}	3.58	36.88^{A}	0.86	38.65^{C}	0.67	22.48^{BC}	1.11
	MH	39.78^{A}	2.03	35.85^{A}	1.01	39.21^{C}	0.91	22.99^{C}	1..46
	H	41.64^{A}	2.79	36.30^{A}	1.13	39.45^{C}	0.81	22.46^{BC}	1.30
	P-value	0.528		<0.001		<0.001		0.001	

Continued

0:1	C	54.66^{A}	2.98	58.16^{C}	0.69	25.78^{A}	1.00	13.80^{A}	1.20
	L	54.09^{A}	3.27	48.62^{BC}	5.85	27.04^{AB}	4.78	14.52^{AB}	3.21
	LM	48.14^{A}	5.06	40.10^{AB}	2.57	36.86^{C}	1.96	21.48^{AB}	2.18
	M	44.12^{A}	3.28	34.84^{A}	1.15	39.69^{CD}	0.72	23.97^{B}	1.88
	MH	40.89^{A}	6.37	30.59^{A}	1.34	42.54^{CD}	0.38	25.23^{B}	2.77
	H	37.46^{A}	7.52	29.94^{A}	1.56	43.98^{D}	0.63	24.60^{B}	3.94
	P-value	0.403		<0.001		<0.001		0.063	

Table S2. Effect of inclusion levels of *Asparagopsis* (C, L, LM, M, MH, H) on volatile fatty acid profile at different Rhodes grass (RG) to barley grain (BG) ratios (1:0, 3:1, 1:1, 1:3, 0:1) after 48 h *in vitro* fermentation. Means with different letters within a column differ significantly at $P < 0.05$.

Diet (RG:BG)	*Asparagopsis* inclusion level	Total VFA (mM)		AA (%)		PA (%)		BA (%)	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
1:0	C	52.09^{A}	4.36	72.70^{C}	0.63	18.84^{A}	0.45	4.82^{A}	0.13
	L	46.96^{AB}	3.76	59.46^{B}	2.72	25.39^{B}	1.73	9.23^{B}	0.55
	LM	41.10^{AB}	0.31	53.27^{A}	0.41	28.73^{C}	0.16	11.08^{BC}	0.42
	M	38.06^{B}	6.59	52.72^{A}	1.91	32.07^{D}	0.98	10.93^{BC}	1.55
	MH	42.39^{AB}	2.79	50.38^{A}	0.89	33.47^{DE}	0.70	12.44^{C}	0.60
	H	39.68^{AB}	3.19	49.34^{A}	0.84	35.79^{E}	0.45	12.33^{C}	0.59
	P-value	0.247		<0.001		<0.001		<0.001	
3:1	C	61.20^{A}	5.20	68.62^{D}	0.95	18.50^{A}	0.76	8.79^{A}	0.27
	L	55.37^{A}	10.7	59.53^{C}	3.52	22.67^{B}	2.30	11.66^{B}	0.46
	LM	58.38^{A}	1.04	50.14^{B}	0.91	27.43^{C}	0.87	16.18^{C}	0.09
	M	50.43^{A}	4.30	46.20^{AB}	0.57	32.45^{D}	0.56	17.17^{C}	0.86
	MH	48.78^{A}	3.83	44.17^{A}	0.92	34.69^{DE}	0.28	18.00^{C}	1.01
	H	47.35^{A}	4.56	42.57^{A}	0.72	36.19^{E}	0.29	18.34^{C}	0.71
	P-value	0.344		<0.001		<0.001		<0.001	
1:1	C	60.17^{A}	7.21	66.09^{D}	0.51	18.99^{A}	0.46	10.71^{A}	0.16
	L	58.63^{A}	3.91	55.64^{C}	2.64	23.29^{B}	1.65	14.99^{B}	0.90
	LM	54.61^{A}	4.69	45.66^{B}	0.60	29.51^{C}	1.34	18.77^{C}	0.42
	M	50.47^{A}	8.60	44.21^{B}	1.64	33.97^{D}	1.15	18.81^{C}	1.58
	MH	50.29^{A}	7.73	41.21^{AB}	0.99	35.38^{D}	1.39	20.55^{C}	0.64
	H	49.43^{A}	5.82	40.00^{A}	1.24	36.04^{D}	1.13	21.16^{C}	0.70
	P-value	0.771		<0.001		<0.001		<0.001	

Continued

1:3	C	68.95^{A}	6.55	61.89^{D}	0.67	21.89^{A}	0.89	11.99^{A}	0.42
	L	65.39^{A}	7.68	49.53^{C}	4.55	26.67^{B}	2.06	17.11^{B}	2.04
	LM	59.26^{A}	5.79	41.36^{B}	0.51	32.17^{C}	0.56	21.00^{C}	0.80
	M	55.43^{A}	5.83	38.13^{AB}	0.86	35.98^{CD}	1.31	22.61^{C}	0.87
	MH	53.59^{A}	5.05	35.79^{AB}	1.01	37.66^{D}	1.25	24.43^{C}	0.44
	H	52.45^{A}	5.87	34.65^{A}	1.13	38.56^{D}	1.52	23.85^{C}	0.75
	P-value	0.376		<0.001		<0.001		<0.001	
0:1	C	70.53^{A}	9.04	59.32^{B}	0.69	22.98^{A}	0.63	13.49^{A}	0.44
	L	77.59^{A}	3.00	50.85^{B}	5.85	23.66^{A}	3.34	14.62^{A}	2.30
	LM	64.29^{A}	6.65	39.62^{A}	2.57	33.91^{B}	2.35	21.70^{B}	1.24
	M	55.81^{A}	9.07	34.84^{A}	1.15	38.12^{B}	1.86	24.08^{BC}	0.89
	MH	57.21^{A}	5.11	32.34^{A}	1.34	39.22^{B}	1.72	25.38^{BC}	0.67
	H	52.09^{A}	7.51	31.05^{A}	1.56	40.45^{B}	2.16	26.01^{C}	0.96
	P-value	0.447		<0.001		<0.001		<0.001	