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Exploration of Methane Mitigation Efficacy Using *Asparagopsis*-Derived Bioactives Stabilized in Edible Oil Compared to Freeze-Dried *Asparagopsis in Vitro*

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Abstract

Asparagopsis oil products are of interest due to the stabilizing effects of the *Asparagopsis*-derived antimethanogenic bioactive compound bromoform ($CHBr_3$). The objective of this *in vitro* series is to characterize antimethanogenic efficacy of freeze-dried *Asparagopsis* (FD-Asp) and *Asparagopsis* oil (Asp-Oil) and compare relative antimethanogenic response over time at multiple levels of $CHBr_3$ delivery. Relative methane (CH_4) emissions (mL/g) are based on *in vitro* apparent feed digested dry matter (IVDDM) after 24, 48, and 72 h of fermentation. $CHBr_3$ contained in FD-Asp was included at 95, 191, and 286 mg/kg, and $CHBr_3$ contained in Asp-Oil was included at 78, 117, and 175 mg/kg, to produce the Low, Mid, and High inclusions, respectively. Low FD-Asp had no significant impact on CH_4 emissions, Mid FD-Asp demonstrated 91%, 44%, and 37% reductions, and the High FD-Asp demonstrated complete inhibition of CH_4, after 24, 48, and 72 h of fermentation, respectively. Comparatively, Low Asp-Oil demonstrated a 46%, 28%, and 18% CH_4 reduction, Mid Asp-Oil resulted in 99%, 92%, and 73% reductions, and the High Asp-Oil demonstrated complete inhibition of CH_4 after 24, 48, and 72 h of fermentation, respectively. IVDDM and total volatile fatty acid (tVFA) production were not changed by the inclusion of FD-Asp and Asp-Oil. The results from this study show that *Asparagopsis* is not only a compelling CH_4 mitigating feed supplement but is also able to be delivered in edible oil forms which

will strengthen its applicability to on-farm use. This study is promising for the utility of Asp-Oil, and *in vivo* trials are essential to demonstrate the extent of efficacy of Asp-Oil in ruminant animals because FD-Asp has consistently demonstrated greater antimethanogenic efficacy *in vivo* compared to *in vitro*.

Keywords

Asparagopsis, Edible Oil, Methane, Greenhouse Gas Mitigation, *In Vitro*

1. Introduction

Globally, burdening greenhouse gas (GHG) inventories remain a prominent challenge, and endeavors to limit GHG emissions are intensifying in most sectors. An important focus of GHG management in the agriculture sector has been enteric methane (CH_4) emissions a hallmark of the red meat, dairy, and wool production industries that extends to the wider agriculture sector because of the pervasive level of this GHG source [1]. Techniques to mitigate enteric CH_4 emissions from livestock production, particularly antimethanogenic feed modification, are evolving to be effective, practical, and increasingly of interest [1] [2]. Freeze-dried *Asparagopsis* spp. (FD-Asp) has been confirmed as a potent antimethanogenic feed ingredient in ruminant diets [3] [4] attributed to the natural biosynthesis of antimethanogenic metabolites dominated by the *Asparagopsis* natural bioactive bromoform ($CHBr_3$) [5]. That said, the evolution of techniques and stabilized products for delivery of *Asparagopsis* bioactive compounds to livestock is imperative for comprehensive CH_4 abatement in variable feeding systems including grazing systems.

The application of edible oils as a stable carrier is emerging as an effective technique, although such activity requires proof of concept and knowledge of minimum effective inclusion levels (MEIL) using variable feeding strategies and demonstration of stability and shelf life. Reference [6] reported degradation of *Asparagopsis* antimethanogenic efficacy with changes in dewatering techniques, this induced research into low-cost stabilization that resulted in experimentation in *Asparagopsis* suspension in edible oils [7]. Steeping *Asparagopsis* spp. in edible oils (Asp-Oil) results in stabilization of the seaweed-derived $CHBr_3$ that fits well with total mixed rations (TMR) where edible oils are already a functional feed ingredient. Controlling the $CHBr_3$ content of Asp-Oil is feasible and crucial, particularly for supplement formulation due to limited oil loading capacity. In this *in vitro* first assessment, if Asp-Oil is proven to perpetuate the antimethanogenic efficacy demonstrated by FD-Asp [8] [9] then *in vivo* animal assessment would follow with expectations that antimethanogenic efficacy would perpetuate in animals in the same way as demonstrated with FD-Asp [3] [4]. Edible oils are already an intrinsic part of intensive animal production systems, such as dry lot dairy and beef feedlots. It is expected that this would support a seamless,

cost-reduced, and stabilized entry of *Asparagopsis* antimethanogenic efficacy delivered in Asp-Oil. Subsequent work to prove the concept in supplements common in extensive rangeland systems is also required and stabilized *Asparagopsis* bioactive content will be required to deliver the MEIL in concentrated pulse delivery. Development of techniques to deliver, encourage animal intake, and stabilize the bioactive compounds of *Asparagopsis* spp. products need to be inherently inclusive of the dominant production systems, including extensive rangeland systems, as is necessary for maximal distribution and environmental benefits. This is crucial for achieving emissions reductions at levels to achieve carbon-neutral red meat and dairy products in this decade [10]. This is particularly important for regions such as Australia, where the red meat, dairy, and wool industries, by animal head count, are largely a pastoral industry [11] and enteric CH_4 contributions are responsible for almost 10% of the country's total emissions [12].

If *Asparagopsis* is to be used as a feed ingredient for ruminant livestock, the contribution of $CHBr_3$ to stratospheric ozone depletion potential (ODP), due to large-scale emissions from ocean and land-based aquaculture of *Asparagopsis* at a large scale, needs to be considered [13]. Geoscientists at the GEOMAR Holtz Center for Ocean Research (Germany), the Institute of Space and Atmospheric Studies (Canada), and the Norwegian Institute for Air Research (Norway) combined to study potentials for $CHBr_3$-specific halomethane emissions from *Asparagopsis* cultivation and reported negligible contribution to ODP [13]. However, the researchers also recommended that losses of $CHBr_3$ to the atmosphere during processing, specifically dewatering, could be beneficially reduced, and to that end, Asp-Oil has been demonstrated to conserve $CHBr_3$ during processing and storage [7].

This study investigates the *in vitro* antimethanogenic efficacy of Asp-Oil and its subsequent impact on feed digestibility and rumen fermentation compared to its counterpart FD-Asp and is the first assessment of Asp-Oil in rumen fermentation. Published research indicates there are variable levels of $CHBr_3$ content in FD-Asp and subsequent inconsistency in product quality due to inherent variability in *Asparagopsis* biomass [14] and $CHBr_3$ losses during processing [6]. Inconsistency may be eliminated with an Asp-Oil product and allows for standardized product formulation to streamline incorporation into commercial products for delivery in bulk. The hypothesis is that Asp-Oil will be equally effective at enteric CH_4 abatement compared to the benchmark FD-Asp with no negative impacts on rumen fermentation or feed substrate digestibility. Specific objectives include: 1) quantification of *in vitro* total CH_4 production per unit of digested dry matter (mL/g); 2) characterization of the *in vitro* MEIL required to reduce CH_4 production below the limit of detection using Asp-Oil of known $CHBr_3$ content; and 3) characterization of the effects of objectives 1) and 2) on rumen fermentation based on substrate digestibility and volatile fatty acid (VFA) profiles.

2. Materials and Methods

2.1. Preparation of Feed Substrate, Freeze-Dried *Asparagopsis*, and *Asparagopsis* Oil

Irrigated Rhodes grass (RG; Chloris gayana) was used as the *in vitro* feed substrate. Preparation of substrate included drying for 24 h at 70˚C, grinding to pass a 1 mm sieve, and was included for rumen fermentation at 1 g OM (1.16 g DM). The RG was analyzed for dry matter (DM), and organic matter (OM) as described by reference [15] by drying to constant weight at 105˚C, and determination of loss on combustion after 8 h at 550˚C, respectively. Ankom Technology model 200 fiber analyzer (Macedon, NY, USA) was used to determine neutral and acid detergent fiber, and crude protein was determined using a LECO model CHN628 nitrogen analyzer (St. Joseph, MI, USA). Analysis results are tabulated in Table 1. The FD-Asp product and its counterpart Asp-Oil were provided by the Centre for Macroalgal Resources and Biotechnology (MACRO) of James Cook University, Townsville, Queensland, Australia. The *Asparagopsis* biomass was sourced from Magnetic Island, Queensland, Australia (19˚12'00.0"S, 146˚49'01.2"E). The FD-Asp and Asp-Oil were prepared for *in vitro* experimentation according to protocols described in detail by references [7] [8]. Briefly, immediately after collection *Asparagopsis taxiformis* in the gametophyte stage was blotted dry, separated into two portions, and frozen on dry ice then subsequently stored at −20˚C until processing. A 1.0 kg portion of fresh weight was subsequently freeze-dried in a benchtop freeze drier (SP Industries VirTis K, Warminster, PA, USA) and milled to pass a 1 mm sieve. Another 1.0 kg portion was added to 1.0 L of rice bran oil (Alfa One, Hansell's Food Group, Condell Park, NSW, Australia), homogenized in a kitchen blender (Anko BL1015-GS, Mulgrave, Vic, AUS) and allowed to steep for 12 days at 4˚C. The FD-Asp and Asp-Oil products were stored at −20˚C until the initiation of the *in vitro* study. The day prior to initiation of each *in vitro* incubation experiment, the FD-Asp and Asp-Oil were brought to room temperature and representative portions weighed into respective fermentation vessels according to the requirements of the treatment groups (Table 2).

Table 1. Dry matter, organic matter, and bromoform content of Rhodes grass, freeze-dried *Asparagopsis* (FD-Asp) and rice bran oil *Asparagopsis* oil (Asp-Oil) (g/kg DM unless stated otherwise).

Composition	Rhodes Grass	FD-Asp	Asp-Oil
Dry matter (g/kg as fed)	927	951	945
Organic matter	803	398	957
Crude Protein	170	90	-
Neutral detergent fiber	648	-	-
Acid detergent fiber	308	-	-
Bromoform (mg/g as fed)	-	18.2	3.69

Table 2. Inclusion levels of Rhodes grass (RG), freeze-dried *Asparagopsis* (FD-Asp), *Asparagopsis* oil (Asp-Oil), and the resultant bromoform levels of the treatments in the respective rumen fermentations.

Treatment		Inclusion (g DM)	RG (g DM)	Treatment Inclusion (g/kg DM)	Bromoform inclusion (mg/kg DM)
Control	-	-	1.16	-	-
FD-Asp	Low	0.006	1.16	5.21	95.0
	Mid	0.012	1.16	10.5	190
	High	0.018	1.16	15.7	286
Asp-Oil	Low	0.024	1.16	21.1	78.0
	Mid	0.037	1.16	31.7	117
	High	0.055	1.16	47.5	175

2.2. Donor Animals and Preparation of Rumen Fluid Inoculum

The donor animals were maintained at the Commonwealth Industrial and Scientific Research Organization (CSIRO) Lansdown Research Station near Townsville, Queensland, Australia (19˚39'27.000"S, 146˚50'04.60"E) according to the Australian code for the care and use of animals for scientific purposes [16] and approved by the CSIRO animal ethics committee (Ethical Clearance Certificate 2018-37). Rumen fluid (RF) was collected and processed for *in vitro* experimentation as previously described by reference [8]. Briefly, four fistulated Brahman steers fitted with 10 cm Bar Diamond (Parma, OH, USA) rumen cannulas were used as RF donors. The steers were grazing mixed rangeland of mainly RG and were given ad libitum access to supplementary irrigated RG hay. The steers were brought in from pasture at 6:00 am, and at 8:00 am approximately 1 L of RF was extracted from of each steer and placed into pre-warmed, 1 L insulated thermos bottles. Prior to inoculation of the fermentation vessels the RF was pooled, filtered through a 0.5 mm sieve, then combined with artificial rumen buffer [17] at a ratio of 1 part RF and 4 parts buffer. Subsequently, 125 mL of the RF buffer mixture was added (Dose-It, Integra Biosciences, Hudson, NH, USA) into each of the 250 mL fermentation vessels (Schott AG, Mainz, DE) containing the substrate/treatment combinations. Inoculation was completed with a headspace purge with high purity N2, then sealed with the Ankom RF1 gas production module (Macedon, New York, United States). Approximately 30 s was required for each inoculation and the accumulating time from first vessel to last was accounted in the total gas produced to ensure equivalent fermentation time throughout the full experimental period. Fermentation vessels were placed into Ratek OM11 dry incubators (Boronia, Victoria, Australia) and maintained at a constant temperature of 39˚C and oscillation speed of 85 RPM.

2.3. *In Vitro* Set up and Experimental Design

The two *Asparagopsis* products were used as treatments [RG substrate plus

FD-Asp or Asp-Oil] along with corresponding RG Controls without *Asparagopsis* products. Three inclusion levels of FD-Asp were tested based on weight of FD-Asp and corresponding $CHBr_3$ content identified as Low, Mid, and High representing 95, 191, and 286 mg/kg substrate DM, respectively. Asp-Oil and corresponding $CHBr_3$ content identified as Low, Mid, and High representing 78, 117, and 175 mg/kg substrate DM, respectively. Inclusion levels were previously tested in a smaller exploratory pretrial incubation experiment (data not shown) to standardize CH_4 reduction potentials for Low, Medium, and High inclusions between FD-Asp and Asp-Oil even though $CHBr_3$ content and weights differ between *Asparagopsis* products. All experimental treatments [7 total] were tested in duplicate over 3 fermentation periods: 24, 48, and 72 hours and this was repeated over three separate *in vitro* incubation experiments. Data collected for each treatment, fermentation period, and incubation experiment were then combined and analyzed for time series results.

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 reference [18]. The Ankom *in vitro* system measures TGP by recording cumulative gas pressure and pressure release at the maximum pressure set point of 3 psi, and recorded as cumulative pressure every 20 minutes, for the duration of the experiment. At the end of each fermentation period of 24, 48, or 72 h the representative fermentation vessels were sacrificed for determination of IVDDM of the RG substrate as well as VFA production within the *in vitro* RF as measures of effect of FD-Asp or Asp-Oil on rumen fermentation *in vitro*. Cumulative gas pressure is then converted into TGP using the natural gas law. Preceding sacrifice of fermentation vessels at termination of each fermentation period, headspace gas samples were collected into 10 mL Labco Exetainer vacuum vials (Lam-peter, Great Britain) then analyzed on a Shimadzu GC-2014 Gas Chromatograph (GC) (Kyoto, Japan) operating with limits of detection (LOD) and quantification (LOQ) of 0.001 mL/g and 0.002 mL/g, respectively. Briefly, the GC was equipped with a Restek ShinCarbon ST column [2 m × 1 mm micro-packed 100/120] (Bellefonte, Pennsylvania, United States) and a flame ionization detector (FID). Column parameters were set to 150˚C, injector at 240˚C, and FID at 380˚C. Ultra-high purity N2 was used as the carrier gas with a flow rate of 25 mL/min and total injection volume of 250 µL. *In vitro*, CH_4 production was determined by their respective concentration in headspace gas relative to TGP while assuming constant homogeneity of fermentation headspace and is reported in the results section as mL of CH_4 per g IVDDM.

2.4.2. *In vitro* Rumen Fermentation

After the fermentation vessels were sacrificed [24, 48, or 72 h] they were chilled in a −20˚C cooler, to cease microbial fermentation, then all the *in vitro* RF was

vacuum filtered through a Duran No. 1 (DWK Life Sciences; Mainz, Germany) porosity sintered glass crucible with a 0.5-cm layer of sand filtration aid. The crucible containing the filtered residues remaining after *in vitro* fermentation was then oven-dried at 105˚C until constant weight. The difference in substrate DM added to initiate fermentation, and substrate residue remaining after fermentation, is the measure of IVDDM. During filtration for IVDDM, a 4 mL sample of *in vitro* RF filtrate was collected for quantification of VFA production expressed as total VFA (tVFA) and subsequent subspecies of VFA corresponding to the 24, 48, and 72 h fermentation periods. Major subspecies of acetate, propionate, and butyrate are reported separately as proportions of tVFA (%) while other minor subspecies are accounted only as part of tVFA. The filtrate sample was added to 1 mL of 20% metaphosphoric acid with an internal standard concentration of 11 mM of 4-methylvaleric acid (Sigma-Aldrich; Castle Hill, NSW, Australia), then stored at −20˚C until analysis. For analysis, a 1.5 mL thawed subsample of filtrate was centrifuged at 13,500 g at 4˚C for 15 minutes, the supernatant was filtered through a 0.2 mm PTFE syringe tip filter (Agilent; Santa Clara, CA, USA) and then analyzed by a Shimadzu GC-2010 Gas Chromatograph equipped with a Restek Stabilwax fused silica column [30 m × 0.25 mm × 0.25 mm] and FID. Column temperature started at 90˚C then ramped up to 155˚C, using 3˚C increases per minute, and held constant for 8.3 minutes. Injector and FID temperatures were held constant at 220˚C and 250˚C, respectively. Ultra-high purity N2 was used as the carrier gas with a flow rate of 1.5 mL/min and total injection volume of 1.0 µL.

2.4.3. Statistical Analysis

Replicate fermentation vessels within the three separate *in vitro* incubation experiments at the three fermentation time series periods (24, 48, 72 h) were first averaged then used as treatment means (Control, Low, Mid, High) for statistical analysis (n = 3). The mean effects of FD-Asp and Asp-Oil inclusion levels were analyzed for TGP, CH_4, IVDDM, and VFA production. Differences among means were tested by one way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) procedure of SPSS Statistics 27 (IBM Corp, Armonk, NY, USA). Effects were declared significant at $P < 0.050$ and $P = 0.050 – 0.100$ were considered as a trend.

3. Results

3.1. Total Gas and Methane Production

Difference in TGP production (mL/g IVDDM) during simulated rumen fermentations *in vitro* as induced by FD-Asp and Asp-Oil at gradient inclusion levels is displayed as response after 24 h, 48 h and 72 h incubation periods (**Figure 1**). Compared to Control, TGP was not impacted by Low FD-Asp after 24 h of fermentation ($P = 0.354$), however Mid and High FD-Asp decreased TGP by 16% ($P = 0.003$) and 17% ($P < 0.001$), respectively. Comparatively, TGP was reduced compared to Control, with Low, Mid, and High Asp-Oil inclusion by 8%

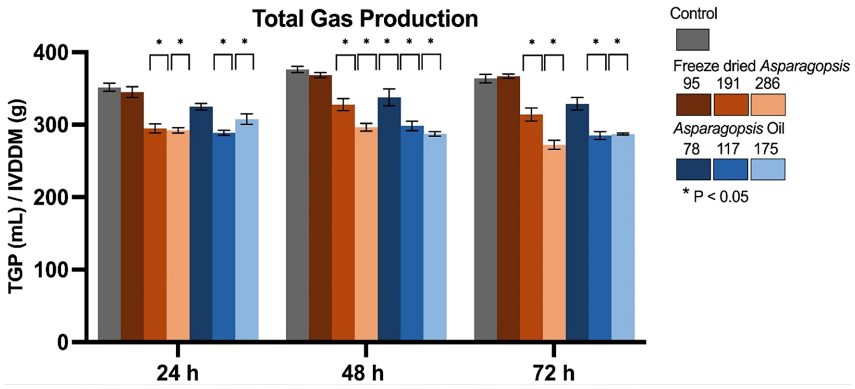

Figure 1. Total Gas Production (TGP) (mL/g IVDDM) after 24, 48, and 72 h of fermentation for Control and three bromoform inclusions (Low, Mid, and High) delivered as freeze-dried *Asparagopsis* or *Asparagopsis* oil. Inclusion levels are expressed as bromoform per unit of substrate dry matter (mg/kg DM).

(P = 0.252), 18% (P < 0.001), and 13% (P = 0.001), respectively. Similar trends in TGP were observed after 48 h of fermentation with no changes for Low FD-Asp (P = 0.352), however reductions of 13% (P < 0.001), and 21% (P < 0.001) were observed for Mid and High FD-Asp, respectively. Low, Mid, and High Asp-Oil demonstrated further significant decreases after 48 h of fermentation by 10% (P = 0.004), 21% (P < 0.001), and 24% (P < 0.001), respectively. After 72 h of fermentation, FD-Asp results were consistent with effects seen after 24 h and 48 h with no difference in Low (P = 0.322), 14% decrease in Mid (P < 0.001), and 25% decrease in High (P < 0.001) FD-Asp inclusions. Consistency was also observed after 72 h of fermentation for Asp-Oil with only minor differences relative to the 48 h TGP observed, compared to Control, with 10% (P = 0.070), 22% (P < 0.001), and 21% (P < 0.001) reductions for Low, Mid, and High Asp-Oil inclusions, respectively. Addition of FD-Asp and Asp-Oil universally reduced TGP which was always significant when induced by the Mid and High inclusion levels and was sustained over time.

The antimethanogenic similarity between FD-Asp and Asp-Oil was elucidated by in-creasing their respective $CHBr_3$ delivery rates and monitoring the CH_4 reduction over 72 h of fermentation. Increasing inclusion level was demonstrated as the prominent influencing factor in efficacy of CH_4 reduction and increased the magnitude and consistency over time. The CH_4 yield (mL/g IVDDM) for Control and Low, Mid, and High inclusion levels of FD-Asp and Asp-Oil after 24, 48, and 72 h of *in vitro* fermentation are presented in **Figure 2**. Percent CH_4 reductions for FD-Asp and Asp-Oil inclusions, compared to Control, are shown in **Table 3**. After 24 h of fermentation, Low FD-Asp did not significantly impact CH_4, however significant reductions were observed with Mid and High FD-Asp inclusions resulting in 9% (P = 0.410), 91% (P < 0.001), and 100% (P < 0.001) CH_4 reductions, respectively. Comparatively, Asp-Oil significantly reduced CH_4 emissions at all three levels of inclusion (P < 0.001). Low Asp-Oil resulted in 45% CH_4 reduction whereas the Mid and High Asp-Oil inclusions resulted in mitigation of CH_4 to levels below LOD, for essentially 100% CH_4 inhibition.

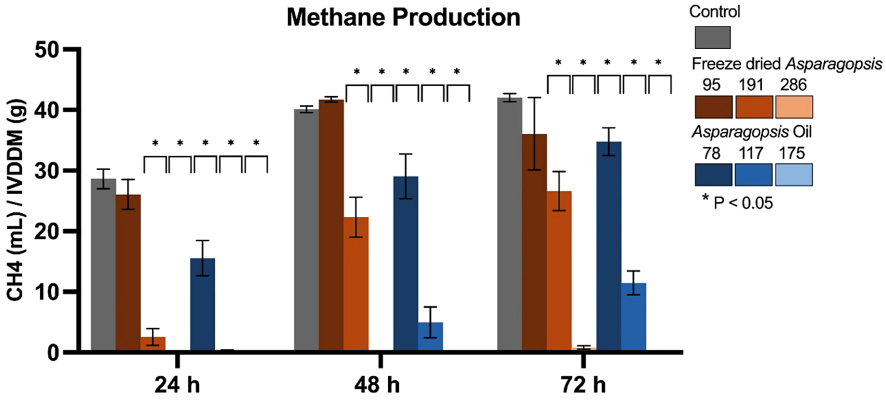

Figure 2. Methane Production (CH_4) (mL/g IVDDM) after 24, 48, and 72 h of fermentation for Control and three bromoform inclusions (Low, Mid, and High) delivered as freeze-dried *Asparagopsis* or *Asparagopsis* oil. Inclusion levels are expressed as bromoform per unit of substrate dry matter (mg/kg DM).

Table 3. Reductions in CH_4 production (%) after 24, 48, and 72 h of fermentation induced by the Low, Mid, and High inclusions of freeze-dried *Asparagopsis* (FD-Asp) and *Asparagopsis* oil (Asp-Oil). Inclusion levels in brackets levels are expressed as bromoform per unit of substrate dry matter (mg/kg DM). The brackets indicate the amount of bromoform (mg/kg DMI) for low, mid, and high inclusions of Asp-Oil.

Treatment		24 h	P	48 h	P	72 h	P
FD-Asp	Low [95]	9.26	0.410	−4.2	0.602	−4.8	0.530
	Mid [191]	91.1	<0.001	44.3	<0.001	37.0	<0.001
	High [286]	100	<0.001	100	<0.001	98.0	<0.001
Asp-Oil	Low [78]	45.6	<0.001	27.9	0.001	17.5	0.026
	Mid [117]	99.0	<0.001	92.4	<0.001	72.9	<0.001
	High [175]	100	<0.001	100	<0.001	100	<0.001

Reductions in CH_4 followed the same pattern when extended to 48 h of fermentation, although were slightly less effective with the extended time. Compared to Control, Low FD-Asp CH_4 was not different ($P = 0.602$), however the Mid and High FD-Asp inclusions resulted in significant reductions in CH_4 by 44% ($P < 0.001$) and 100% ($P < 0.001$), after 48 h of fermentation, respectively. Additionally, while Mid FD-Asp worked to reduce CH_4 emissions by 91% over 48 hours, the efficacy of CH_4 mitigation was diminished to 44% over 48 h of fermentation. Comparatively, after 48 h of fermentation, the Asp-Oil significantly reduced CH_4 production by 28% ($P = 0.001$), 92% ($P < 0.001$), and 100% ($P < 0.001$) for Low, Mid, and High inclusions, respectively. Similar to Mid FD-Asp but to a lesser degree, the Mid Asp-Oil slightly diminished in CH_4 mitigation ability over time with 100% and 92% reductions after 24 h and 48 h of fermentation, respectively.

After 72 h of fermentation, Low FD-Asp shows no CH_4 reduction ($P = 0.530$) and Mid FD-Asp demonstrated reduced CH_4 reduction efficacy of 37% ($P <$

0.001). The High FD-Asp maintained a near complete mitigation of 98% after 72 h of fermentation ($P < 0.001$), producing only trace (1 mL/g IVDDM) CH_4 up to termination of each incubation experiment. Asp-Oil demonstrated declining CH_4 mitigation effectiveness for the Low and Mid Asp-Oil inclusion after 72 h of fermentation with 18% ($P = 0.026$) and 73% ($P < 0.001$) reductions, respectively. However, High Asp-Oil continues to completely mitigate CH_4 ($P < 0.001$). With FD-Asp and Asp-Oil a sustained elimination of CH_4 was achieved with the High inclusion level thereby indicating the *in vitro* MIEL for As-paragopsis derived $CHBr_3$ was approaching 286 mg/kg and 175 mg/kg, respectively.

3.2. *In vitro* Rumen Fermentation

The IVDDM remained relatively consistent and independent of inclusion level of FD-Asp or Asp-Oil with digestibility of substrate RG not different or only marginally increased compared to Control throughout 72 h of fermentation (**Figure 3**). That said, marginal in-crease in IVDDM was detected with Low FD-Asp after 24 h and 48 h of fermentation at 5.7% ($P = 0.036$) and 5.1% ($P = 0.032$) increases, respectively. The Mid FD-Asp inclusion also demonstrated marginal IVDDM increase of 5.4% ($P = 0.050$) but only after 24 h of fermentation and was otherwise not significantly different than Control. Low Asp-Oil was at the threshold of demonstrating increased substrate digestion at 72 h of fermentation, with increased IVDDM of 4.3% ($P = 0.050$), however was not significantly different from Control for the first 48 h of fermentation. The inclusion of *Asparagopsis* as FD-Asp or Asp-Oil did not induce negative response in *in vitro* rumen fermentations based on IVDDM. Furthermore, there was universal marginal improvements, and significantly increased IVDDM induced by Low levels of Asp-Oil.

In vitro production of VFAs is a consequence of simulated ruminal fermentation of feed substrate in RF and the VFAs are captured in the *in vitro* RF. Production of tVFA, VFA species (acetate, propionate, and butyrate), and the ratio between acetate and propionate for Control and Low, Mid, and High inclusions of FD-Asp and Asp-Oil after 72 h of fermentation are presented in **Figure 4**. Production of tVFA remained relatively stable with increasing levels of *Asparagopsis*-derived $CHBr_3$ when delivered in FD-Asp and Asp-Oil products. There was no significant difference in tVFA between control compared to any level of FD-Asp or Asp-Oil after 72 h of fermentation. That said, there was marginal numerical differences visible in **Figure 4** such that Low FD-Asp induced a 2% ($P = 0.854$) increase in tVFA however all other treatments marginally reduced tVFA. The Mid, and High FD-Asp induced marginal decreases in tVFA of 7% ($P = 0.401$), and 10% ($P = 0.244$) compared to Control, respectively. The Low and Mid Asp-Oil induced marginal reductions in tVFA by 5% ($P = 0.748$), 11% ($P = 0.232$), respectively. However, High Asp-Oil induced a trend toward reduced tVFA of 17% ($P = 0.061$) compared to Control.

Acetate production which is expected to increase with time and IVDDM also significantly decreased as a result of increasing levels of FD-Asp and Asp-Oil

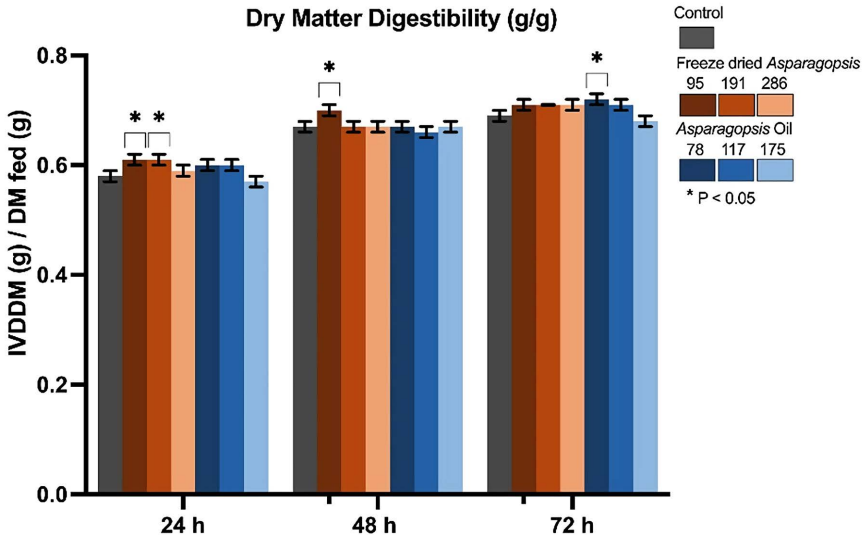

Figure 3. Apparent *in vitro* digestibility of substrate dry matter (IVDDM) (g/g DM fed) after 24, 48, and 72 h of fermentation for Control and three bromoform inclusions (Low, Mid, and High) delivered as freeze-dried *Asparagopsis* or *Asparagopsis* oil. Inclusion levels are expressed as bromoform per unit of substrate dry matter (mg/kg DM).

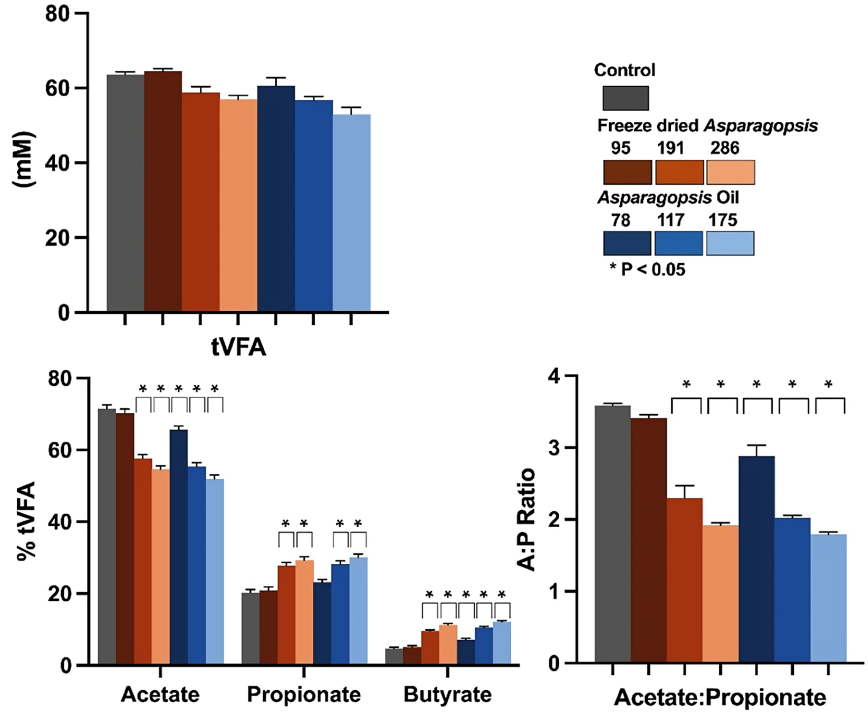

Figure 4. Production total volatile fatty acid (tVFA), acetate, propionate, and butyrate as a proportion of tVFA, and acetate to propionate ratio (A:P) after 24, 48, and 72 h of fermentation for Control and three bromoform inclusions (Low, Mid, and High) delivered as freeze-dried *Asparagopsis* or *Asparagopsis* oil. Inclusion levels are expressed as bromoform per unit of substrate dry matter (mg/kg DM).

with the exception of FD-Asp Low inclusion. Low FD-Asp resulted in no significant changes ($P = 0.485$) in the tVFA proportion of acetate compared to Con-

trol for the full 72 h of fermentation. FD-Asp resulted in decreased acetate by 19% ($P < 0.001$) and 24% ($P < 0.001$) for Mid and High inclusion groups, respectively. All levels of inclusions of Asp-Oil resulted in changes in acetate compared to Control after 72 h of fermentation by 8% ($P < 0.001$), 22% ($P < 0.001$), and 27% ($P < 0.001$) for Low, Mid, and High inclusion groups, respectively.

An inverse relationship between acetate and propionate production resulted in elevated propionate as a proportion of tVFA. Low FD-Asp resulted in no significant changes ($P = 0.666$) in propionate compared to Control for the full 72 h of fermentation. Mid and High FD-Asp inclusions resulted in increased propionate by 37% ($P < 0.001$) and 45% ($P < 0.001$) after 72 h of fermentation, respectively. Comparatively, Low Asp-Oil resulted in a trend of increased propionate by 14% ($P = 0.078$) whereas Mid and High resulted in significant changes of 39% ($P < 0.001$) and 48% ($P < 0.001$), respectively, after 72 h of fermentation.

Butyrate production, as a proportion of tVFA, was significantly and greatly increased with increasing levels of FD-Asp and Asp-Oil. Low FD-Asp resulted in small, nonsignificant changes in butyrate of approximately 10% ($P = 0.377$) compared to Control after the full 72 h of fermentation. The Mid and High FD-Asp inclusions were demonstrated to induce increases of 103% ($P < 0.001$) and by 139% ($P < 0.001$) compared to Control, after 72 h of fermentation, respectively. All inclusion levels of Asp-Oil resulted in increased butyrate compared to Control after 72 h of fermentation. Low, Mid, and High Asp-Oil inclusions resulted in 52% ($P < 0.001$), 124% ($P < 0.001$), and 158% ($P < 0.001$) increases in butyrate, respectively.

Responses of decreased acetate and increased propionate production, induced by increasing levels of *Asparagopsis*-derived $CHBr_3$, almost universally reduced the acetate to propionate ratio (A:P). Compared to Control, the only nonsignificant decrease ($P = 0.403$) in A:P was seen in the Low FD-Asp inclusion. Mid FD-Asp resulted in decreased A:P by 36% ($P < 0.001$) and High FD-Asp resulted in decreased A:P by 47% ($P < 0.001$) after 72 h of fermentation. Comparatively, all levels of inclusion of Asp-Oil resulted in decreased A:P, compared to Control. Low, Mid, and High Asp-Oil resulted in 20% ($P = 0.002$), 44% ($P < 0.001$), and by 50% ($P < 0.001$) decrease in A:P after 72 h of fermentation.

4. Discussion

4.1. Total Gas and Methane Production

Variability in TGP correlates proportionately to CH_4 reduction *in vitro* ($r^2 = 0.829$) (**Figure 1** & **Figure 2**). The two dominant component gases that are produced, eructated, and respired from ruminants in normal feeding scenarios are carbon dioxide (CO_2) and CH_4. Thus, when CH_4 is significantly reduced it would be expected that TGP would also decrease. This result has been recorded in nearly all studies using FD-Asp as a dietary antimethanogenic ingredient *in vitro* [8] [9] [19] [20] [21] and in this study, with both FD-Asp and Asp-Oil, contributing to the consistent trend of TGP decreasing proportionally with CH_4 de-

crease in the same way as induced by virtually all enteric CH_4 inhibitors.

Using FD-Asp as the benchmark, and using $CHBr_3$ content as the measure for efficacy, Asp-Oil was demonstrated *in vitro* to be as effective as FD-Asp at the inclusion levels tested in this study. Compared to FD-Asp, antimethanogenic efficacy was not impaired on a $CHBr_3$ delivery bases when delivered as Asp-Oil under closed system *in vitro* conditions. However, caution is necessary when considering comparison to *in vivo* and will need to be confirmed in animals, especially considering the strikingly increased efficacy of FD-Asp demonstrated with transition from *in vitro* [9] to *in vivo* [3]. That said, in this *in vitro* study Asp-Oil suggests increased efficacy for reducing CH_4 yield (mL/g IVDDM), and the MEIL for Asp-Oil delivery of $CHBr_3$ inclusion was up to 39% lower compared to FD-Asp. Considering the nature of FD-Asp which is high density dry biomass of ~50% mineral ash and thus comes with inherently large potential to be poorly homogenous, coupled with small biomass inclusion (<0.02 g) *in vitro*, accuracy of $CHBr_3$ delivery is more difficult than for the highly homogenous low ash liquid Asp-Oil. Therefore, it is more difficult to confirm $CHBr_3$ delivery with consistency using FD-Asp and leads to speculation that lower than target inclusion levels may have been delivered.

Once in the rumen, physical and biochemical factors may also influence the comparative efficacy of FD-Asp and Asp-Oil. The FD-Asp may have moderately impeded capacity to incorporate into a homogeneous digesta as a dehydrated biomass with a propensity for high degree of rehydration (data not shown), thus providing a partial barrier to release of FD-Asp encapsulated $CHBr_3$. Subsequently, this could reduce contact of the antimethanogenic $CHBr_3$ with the target methanogenic archaea in buffer diluted RF. Conversely, Asp-Oil may have improved ability to be incorporated into a homogeneous digesta as a fluid with increased miscibility in the warm rumen fluid. This could increase the tendency for Asp-Oil carried $CHBr_3$ to contact the target archaea throughout the rumen digesta. Free movement within the *in vitro* RF buffer mixture may increase penetration of $CHBr_3$ into vacuoles, lysed cells, and protozoa where some archaea populations inhabit [22]. Additionally, Asp-Oil stabilizes $CHBr_3$ [7] which will improve the overall shelf life of *Asparagopsis* products for commercialization. In house experimentation has demonstrated that $CHBr_3$ content in Asp-Oil is stable between 20˚C - 40˚C for storage of up to 24 weeks (data not shown) and more detailed work on shelf-life stability under a variety of storage conditions is warranted to further elucidate the utility of Asp-Oil compared to FD-Asp.

This study incorporated 100% Rhodes grass as the feed substrate with decisive results suggesting utility in grazing and grass-fed systems, however Asp-Oil has not been demonstrated for a TMR and of particular interest is efficacy in feedlot feed formulations where edible oils are routinely added, thus providing an inherent vector for delivery of Asp-Oil. Alternatively, FD-Asp was also tested *in vitro* using Rhodes grass substrate with decisive results [8] [9] and subsequently, FD-Asp demonstrated excellent efficacy *in vivo* in high grain TMRs [3] [4]. It has been demonstrated that changing the proportion of grass to grain in feed

formulations has significant impact on the *in vitro* antimethanogenic efficacy in favor of increasing the grain content, however complete CH_4 inhibition was demonstrated independent of diet formulation [18]. The preceding studies provide confidence that Asp-Oil antimethanogenic efficacy can be translated to all diet formulations albeit with variability in the MEIL influenced by grass and grain proportions. This is expected to translate to marginally more *Asparagopsis* product required to define the *in vivo* MEIL for grazing systems. Likewise, it remains to be demonstrated if delivery of the *Asparagopsis*-derived bioactives as FD-Asp or Asp-Oil differs in antimethanogenic efficacy *in vivo*. Differences can be expected relative to high grain, grazing concentrate supplemented, or pure grazing systems. These diets are the hallmark of feedlot, dairy, and pastoral systems but mixed systems including silage TMR systems are also prevalent globally. That said, FD-Asp has previously been demonstrated to be universally effective [3] [4] [23]. However, pure grazing systems are a complicated prospect for consistent delivery of *Asparagopsis* products but are the target of ambitious research influenced by the dominant numbers of livestock on grasslands in Australia [24].

The antimethanogenic potential of *Asparagopsis* has been consistently demonstrated both *in vitro* [8] [20] [21] and *in vivo* [3] [4] [25] [26]. Interactions between the *Asparagopsis* components, the carrier oil, and the rumen microbiome may beneficially contribute to antimethanogenic efficacy of Asp-Oil. When dietary lipids, via the addition of vegetable or seed oils, are included in the diet at low levels they can provide small to moderate reductions in enteric CH_4 emissions between 10% - 25% [27] but is also known to confound digestibility of forage-based diets through several mechanisms. Dietary lipids can reduce CH_4 emissions through adsorption onto microbial cell membranes, which disrupts energy metabolism and nutrient transport leading to heightened rumen microbial mortality [28]. Microbial populations most sensitive are those associated with fiber degradation, such as fibrolytic bacteria and ciliate protozoa [29], which reduces the primary CH_4 precursors of CO_2 and hydrogen (H_2) and their subsequent availability for methanogenic archaea [30]. Additionally, lipids have also been reported to lead to direct reductions in methanogenic archaea populations [31], which may be supported through the adsorption pathway or through redirection of H_2 utilization via biohydrogenation of unsaturated fatty acids and increased propionate production [32] [33] [34]. Rice bran oil, the carrier oil used in this study, has been found to reduce enteric CH_4 emissions by almost 14% when fed at 4% inclusion to lactating dairy cows [35]. Those studies report on antimethanogenic benefits in feeding oils but also the potential for reduced feed digestibility, particularly grass. This highlights the need for knowledge on sensitivity of using Asp-Oil in grazing systems and potentially developing stable products for grazing systems with both FD-Asp and Asp-Oil. The objective is to maintain high levels of CH_4 reduction without a negative impact on grass diet digestibility. Likewise, utility in pulse delivery as supplements requires a bioactive concentration in the product that allows for delivery of the MEIL within the

context of limited inclusion bound by factors such as stability, palatability, and small DMI intake as a supplement.

4.2. *In vitro* Rumen Fermentation

It has been hypothesized that large reductions in enteric CH_4 emissions can negatively impact digestibility, mainly due to a buildup of metabolic hydrogen ([H]), in the rumen which in turn has a negative impact on acetate production, the main VFA resulting from fiber digestion [36] [37]. Conversely, previous research has consistently shown that the addition of FD-Asp has no effect on IVDDM [8] [9] [18]. However, an objective of the current study was to determine the comparative impacts of Asp-Oil on IVDDM since the oil carrier itself can impact digestibility and fermentation of substrates in the rumen [28]. To that end, this study demonstrated that both FD-Asp and Asp-Oil have no negative impacts on IVDDM with complete inhibition of CH_4. In addition, decreases in acetate with concomitant increases in propionate and subsequent reduction in acetate:propionate have been consistently demonstrated with FD-Asp *in vitro* [18] [19] [20] and now with Asp-Oil. The phenomena were also observed with FD-Asp *in vivo* [3] [25] and consistency between FD-Asp and Asp-Oil in this study is a strong indicator that Asp-Oil will behave in the same way as FD-Asp *in vivo*.

Increased production of butyrate, on the other hand, was not expected to increase with *Asparagopsis* inclusion. This study shows significant increases in butyrate production and is consistent with previous studies using FD-Asp at different inclusion rates *in vitro* [18] [19]. It has been suggested that when [H] increases in the rumen, some bacterial populations can incorporate [H] when forming butyrate from pyruvate, thus would provide an additional [H] sink in the rumen [38]. The majority of these particular bacteria are of the firmicutes phylum, which has been shown to increase with the addition of *Asparagopsis* [20] [39]. It could be that these specific bacterial populations are also upregulated by the increase in [H] due to CH_4 mitigation, however, it is unclear if this result is also seen *in vivo* thus more research is warranted in this area.

5. Conclusions

This exploratory *in vitro* study has demonstrated compelling outcomes that support Asp-Oil as a candidate with significant potential as the first counterpart to FD-Asp for CH_4 mitigation. Inclusion of Asp-Oil *in vitro* rumen fermentation was shown to be equally antimethanogenic, or possibly more so, compared to the consistent efficacy of FD-Asp. Both *Asparagopsis* products eliminated CH_4 production, however decreasing efficacy was apparent with decreasing inclusion such that over time antimethanogenic efficacy was reduced for the Low and Mid but not for the High level. Furthermore, there was no negative impact of either FD-Asp or Asp-Oil on rumen fermentation parameters of TGP, IVDDM, or VFA after the full 72 h of fermentation. Translation of the *in vitro* proof of con-

cept for Asp-Oil demonstrated successfully in the present study requires demonstration *in vivo*. The utility of FD-Asp in red meat and dairy production has been confirmed for enteric CH_4 mitigation, animal welfare, and food safety in beef cattle [3] [4], dairy cows [23] [25], and sheep [26]. It is expected that Asp-Oil will not be different, however specific studies are required in characteristic feeding scenarios.

This study also showed that *Asparagopsis* can be delivered in the Asp-Oil form, as an alternative to the proven FD-Asp, while still retaining its CH_4 mitigation potential which will diversify its applicability and augment adoption for on-farm use. Some feeding systems already utilize edible oils in the formulation of TMR and supplements thus providing a vector for *Asparagopsis* in an ingredient that may not add cost to the delivery of *Asparagopsis*. That said, the variation that exists in feeding systems calls for innovation to achieve impact at meaningful levels with alternative and advantageous technologies of delivery. Achieving adoption of FD-Asp and Asp-Oil in feeding systems other than TMRs will require evolution to pulse feeding techniques which requires periodic and consistent delivery of a stable supplement containing the *Asparagopsis* product.

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Conflicts of Interest

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

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