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Productivity of Commercial Feedlot Beef Production Significantly Improved by *Asparagopsis* Bioactives Stabilized in Canola Oil

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Abstract

Research using open-circuit respiration chambers has established that *Asparagopsis* bioactive compounds stabilized in canola oil (Asp-Oil), delivering a range of inclusion between 34 - 51 mg bromoform ($CHBr_3$)/kg dry matter intake (DMI), inhibits methane (CH_4) emissions > 98% in feedlot cattle. In this study, Asp-Oil was fed at 35 mg $CHBr_3$/kg DMI in the feedlot finisher diet under highly replicated and commercially relevant conditions, and adequately powered to confirm differences as low as 3.3% in feed conversion efficiency (FCE). The study also evaluated the effect of Asp-Oil on CH_4 production (g/day), carcass and meat-eating qualities, animal health, and food safety. The experiment consisted of 300 Angus-Shorthorn (*Bos taurus*) steers in 30 pens of 10 and fed a barley-based ration supplemented with canola oil (Control, n = 15), or Asp-Oil (n = 15) for total 81-d, inclusive of 21-d transition to full Asp-Oil and grain inclusion in the finisher ration. Reduction of CH_4 was measured using GreenFeed Emissions Monitors (GEM) in a subset of 2 pens in both Control and Asp-Oil. However, 67% of CH_4 measurements were recorded below the GEM's limit of detection. An inhibition range of 58% - 98% was demonstrated by collectively using GEM and preliminary respiration chamber measurements. Asp-Oil improved FCE 7.4% in the finisher diet, and 5.6% across transition and finisher periods. During the transition steps the steers had not yet received their full allocations of grain and Asp-Oil which is responsible for the lower FCE benefits. A 4.1% lower DMI in the finisher period contributed to benefits in FCE with no effect on daily weight gains. Cost of feed and weight gain were reduced $0.35/head/day and $0.23/kg, respectively,

in steers receiving Asp-Oil. Residues of *Asparagopsis* $CHBr_3$ were not detected in any sample and only trace iodide and bromide were detected in livers and kidneys of both Control and Asp-Oil steers at levels safe for human consumption. Steers demonstrated normal rumen development typical of feedlot diets. This study confirms that Asp-Oil safely induces significant productivity benefits and CH_4 reductions in feedlot beef production. Commercial adoption of *Asparagopsis* can benefit feedlot beef production and reduces the climate change contribution of livestock production.

Keywords

Asparagopsis, Bromoform, Enteric Methane, Feed Efficiency, Ruminant, Seaweed

1. Introduction

Asparagopsis spp. has been confirmed as an effective feed ingredient in ruminant diets for reduction of enteric methane (CH_4) emissions in beef [1]-[3] and dairy cattle [4]-[6]. Whilst early research on *Asparagopsis* as a CH_4-inhibitor utilized dried and ground *Asparagopsis* meal (Asp-Meal), recently bioactive compounds of *Asparagopsis* stabilized in canola oil (Asp-Oil), have been demonstrated to be equally as effective *in vitro* [7] and in separate *in vivo* studies in dairy and beef cattle [3] [5]. Currently, techniques for processing fresh *Asparagopsis* to stabilize bioactive secondary metabolites, of which bromoform ($CHBr_3$) is the most abundant, include freeze-drying [8] or steeping in edible oils [9]. Asp-Oil offers improved shelf life in moderately challenging conditions compared to Asp-Meal [10]. In feedlot beef production, addition of oil in the total mixed ration (TMR) is a widespread practice [11], providing a practical advantage for the use of Asp-Oil as an effective CH_4 emissions management tool.

Beef cattle fed high grain diets are of particular interest due to the achievable high level of CH_4 mitigation, which consistently reaches over 98% using respiration chambers as the measurement tool, and at inclusion levels between 25 - 35 mg bromoform ($CHBr_3$)/kg total dry matter intake (DMI) for both Asp-Meal [1] and Asp-Oil [3]. Roque *et al.* [2] reported significant CH_4 mitigation of up to 70% (at 35 mg $CHBr_3$ /kg DMI) and 80% (at 70 mg $CHBr_3$ /kg DMI) in feedlot steers using GreenFeed Emissions Monitor (GEM) units, with Asp-Meal that persisted the duration of a 147-day feeding period. There are a variety of factors that influence CH_4 production, and perhaps mitigation potential of Asp-Meal and Asp-Oil, which may include dietary composition, animal breed and genetics, or a combination of these factors. The techniques used to monitor efficacy of antimethanogenic feed ingredients have evolved to account for the variety of feeding systems globally, to decrease resource requirements, and to accelerate livestock emissions research. The disadvantages inherent in the techniques may contribute to differences in measured antimethanogenic efficacy as intimated by studies of similar

design and feeding protocols. To date, studies with cattle receiving a TMR containing Asp-Meal or Asp-Oil and monitoring emissions using respiration chambers have reported greater than 98% efficacy of CH_4 inhibition [1] [3]. Additionally, using GreenFeed monitoring has not achieved greater than 80% reduction [2] [12]. That said, there are differences between studies that may impact antimethanogenic efficacy that would not be expected to affect efficacy by the observed 20% - 40% discrepancies, thus implicating CH_4 emissions monitoring techniques as a contributor to variation between studies.

It has been demonstrated in small scale feedlot simulation studies that as CH_4 mitigation potential increases, animal productivity characteristics such as live weight gain or feed conversion efficiencies (FCE) may also improve by conserving feed energy otherwise lost in methanogenesis. For example, Kinley *et al.* [1] reported average daily weight gain (ADWG) improvements of 26% with the inclusion of Asp-Meal during a 90-day finishing period while offering the caveat that the number of steers in the treatment groups (n = 5) was too low, and variability too high, to be conclusive on productivity benefits. Still, the study was the first indicator and provided context into potential ADWG improvements. That said, even with significant ADWG improvement, the change in FCE was muted by a 7.5% increase in DMI. Subsequently, Roque *et al.* [2] reported an increase in FCE of up to 21% during the finishing period collateral with a 9.3% increase in ADWG and 7.0% decrease in DMI. Again, significance was muted by low animal numbers in the treatment groups (n = 6), however, a tilt toward productivity gain was beginning to emerge in studies achieving high-level CH_4 mitigation. The benefits of conclusive and significant performance gains, including FCE and ADWG would be considerable for adoption and commercialization of antimethanogenic livestock feeds. Research to date on *Asparagopsis* spp. products have focused on CH_4 mitigation and food safety in mostly small-scale, controlled animal-house experiments, which were not designed or statistically powered to confirm differences in animal productivity and not conducted under commercially relevant conditions [13].

Our hypothesis was that providing Asp-Oil in a TMR to feedlot beef cattle would induce gains in FCE associated with a high level of CH_4 inhibition and do so without detriment to meat quality, including carcass characteristics, sensory attributes, food safety, and animal welfare. The primary objective was to investigate Asp-Oil at the predetermined optimal inclusion of 35 mg $CHBr_3$/kg DMI [3] in a feedlot finisher diet on cattle feedlot and carcass performance under commercial management conditions and with adequately powered and statistically relevant levels of replication. Asp-Oil effect on enteric CH_4 emissions was assessed in a subset of pens using GEM units. A secondary objective was to assess meat and edible offal for residues of *Asparagopsis*, consumer sensory attributes, and indicators of effect on animal health.

2. Materials and Methods

This study was conducted at the University of New England's Tullimba Commercial

Feedlot, New South Wales, Australia (−30.48'S, 151.18'E). The Animal Ethics Committee of the University of New England approved all procedures involved in this experiment (Authority no: ARA22-046).

2.1. Experimental Design

The experimental design followed a randomized block design with 2 dietary treatments nested within 15 initial cattle liveweight blocks. The treatments were comprised of the Control receiving only TMR (containing canola oil) and the Asp-Oil group receiving the same TMR with Asp-Oil substituted for canola oil; both TMR diets contained the same content of oil. Group-fed pens of 10 steers were the experimental unit, and each treatment was replicated in 15 pens (n = 15), which was sufficient to confirm a 3.3% difference in FCE, assuming a 3.2% coefficient of variation, $\alpha = 0.05$ and $\beta = 0.80$ [14].

2.2. Cattle Arrival, Processing, and Treatment Allocation

Five days before the experiment, 300 *Bos taurus* steers of primarily Angus-Shorthorn cross from the same farm were transported to the University of New England's Tullimba Feedlot, weighed and inducted. Induction consisted of pour-on broad-spectrum endectocide (Dectomax® Pour-On, Zoetis, Parsippany, NJ), oral flukicide (Exifluke 240®, Bayer, Leverkusen, Germany), clostridial vaccine (Ultravac® 5in1, Zoetis, Parsippany, NJ), Bovine Respiratory Disease vaccine (Bovilis® MH + IBR, Coopers Animal Health, Macquarie Park, NSW, Australia), and hormonal growth promotant (Component TE-S, Elanco, Macquarie Park, NSW, Australia). Prior to initiation of the study (d −2, −1) the steers were sorted into blocks based on liveweight and were randomly assigned to either the Control or Asp-Oil treatments within each block. The 15 blocks of paired Asp-Oil and Control treatments were randomly allocated into contiguous feedlot pens and adjoining blocked pairs of pens also randomized. Each pen provided 12.5 m^2/head of space and 62.5 cm/head of concrete feed bunk space and was fitted with a reticulated water trough of 90 - 150 cm length.

2.3. Diets and Feeding

Starting on d 0, all steers were fed a tempered barley TMR of the composition described in Table 1. Weekly bulked sub-samples for Starter, Transition I and Transition II diets, and bulked monthly for the Finisher diet for wet chemistry analysis, which is described in detail in Cowley *et al.* [3]. Briefly, 150 g samples of the TMR and refusals were determined for DM content by drying to constant weight at 65˚C. Further subsamples were analyzed for organic matter and ash (ISO 5984:2002[E]), crude fat as ether extract (AFIA Method 1.14R), crude protein (AOAC Method 2001.11), neutral and acid detergent fiber (NFTA Method 008.08 and AAFCO Method 008.08, respectively), starch (AOAC Method 996.11). Total digestible nutrients (TDN) was used to calculate metabolizable energy [15] while TDN (%DM) = 93.50 − (ADF × 0.936) [16].

Table 1. Formulated ingredient composition and analyzed nutrient composition of basal rations fed to the feedlot steers. The canola oil blend was a mix of either Asp-Oil or the Control canola oil.

Item	Diet			
	Starter	Transition I	Transition II	Finisher
Ingredient (g/kg)				
Tempered Barley	485	607	706	802
Oaten hay	159	96.0	59.0	-
Wheat straw	73.0	72.0	47.0	42.0
Millrun	147	84.0	41.0	-
Whole cottonseed	101	99.0	98.0	99.0
Liquid supplement	31.0	35.0	41.0	46.0
Canola oil blend	3.00	6.00	8.00	11.0
Monensin (ppm)	16.3	18.3	21.0	23.6
Analyzed nutrient composition (g/kg)				
Dry Matter	738	742	778	758
Organic Matter	932	947	947	948
Ash	69.0	54.0	54.0	52.0
Crude Protein	125	130	124	121
Fat	41.0	36.0	56.0	50.0
Neutral Detergent Fiber	382	301	314	263
Acid Detergent Fiber	201	139	140	111
Starch	490	453	478	548
Metabolizable Energy (MJ/kg DM)	11.5	12.6	12.6	13.3
Calcium	8.70	6.50	8.70	8.90
Phosphorus	3.60	3.80	3.60	3.70
Potassium	9.60	7.50	7.30	6.40
Magnesium	2.20	2.10	2.10	2.00
Zinc (ppm)	107	96.5	104	125
Bromoform (ppm, Asp-Oil only)	8.80	17.6	26.5	35.3

The grain and canola oil content of the diet was increased in 3 incremental steps through a transition protocol of Starter (5 d), Transition I (5 d) and Transition II (11 d) diets in a ramp-up adaptation process before receiving the final Finisher diet on day 21. All rations for Control and Asp-Oil groups were balanced for canola oil content. The oil in the Control diet was solvent-extracted canola oil and the Asp-Oil contained *Asparagopsis armata* bioactive metabolites stabilized in solvent-extracted canola oil, with residual seaweed biomass removed. Ancillary data and insight were generated for this study during a preliminary experiment, that was designed to quantify and inform on the range of effective inclusion levels

(REIL; [3]). The optimal inclusion level of Asp-Oil $CHBr_3$ was established at 35 mg/kg DMI and reduced emissions of CH_4 by >98% using the gold standard of respiration chambers as the CH_4 emissions monitoring technology. The amount of oil (either canola or Asp-Oil) fed was determined by the amount required to deliver 35 mg $CHBr_3$/kg DMI in the finisher ration. The mother stock of Asp-Oil containing 3.1 mg $CHBr_3$/g canola oil was supplied by Sea Forest Ltd (Triabunna, Tasmania, Australia). The steers were fed once per day at early morning (0700 - 0800 h). A mixer flush consisting of a minimum of two full mixer loads was completed after feedout of the Asp-Oil diet. The oils were stored outside in pallet tanks covered by reflective insulation and mixed by full volume recirculation every week and pumped into the feed mixer (CV < 5%) to provide volumes as required for each ration step.

2.4. Feedlot Performance Characteristics

Daily feed intake was measured and recorded using a bunk-scoring protocol [17] aiming for minimal feed refusals (orts). Each feed bunk was scored before feeding and the new feedout delivered. A weekly bulked sample of daily feed sub-sampled for dry matter (DM) and averaged for both treatments to calculate daily DMI at pen-level.

Liveweight was measured for each animal individually, on d 0 and d 40 during the feedlot period, and on d 81 at termination of the finisher period (feedlot exit) on a calibrated static scale (Gallagher, Hamilton, NZ). Post-feeding, each pen of 10 steers was walked to the weigh-scale and returned to the pen within 30 minutes. The contiguous pen order was always maintained, however, on d 81 was coordinated by treatment for organization of loading cohorts onto transport trucks. The ADWG values were calculated by difference between initial weight and the periodic weight to achieve individual ADWG for d 0 - 40, d 41 - 81, and d 0 - 81. The FCE values were calculated as individual ADWG / pen average per head DMI. The individual ADWG and FCE were averaged within each pen for statistical analysis. Cost of daily feed was calculated per head for each pen, using fixed cost of basal ration only (excluding cost of Asp-Oil). Cost per kg of liveweight gain was calculated on a pen basis, by dividing the cost of feed/head by the cumulative liveweight gain.

2.5. Carcass Performance and Residues

At the conclusion of the finisher period (d 81), all steers were transported to the Australian Country Choice abattoir (Brisbane, Queensland, Australia). The steers were fed before weighing and were kept in their respective pen group when loaded onto a partitioned section of a B-double truck. Each truck deck (upper and lower) contained 3 partitions for the pens and were loaded on as 3 pens of the same treatment balanced across 5 trucks and across truck decks (upper or lower). All pens of steers transported together were slaughtered together, so they were killed in a rotating sequence of 3 Asp-Oil pens, followed by 3 Control pens.

Samples for residue analysis (n = 10) of $CHBr_3$, iodide (I^-) and bromide (Br^-)

were collected as previously described [1] [3]. Briefly, during the process of evisceration in the abattoir, samples of liver and kidney for residue analysis were collected from the hot carcasses. Meat samples of shin/shank muscle (Fore *M. extensor/flexor*) for residue analysis were collected during boning, after chilling 18 hours at 5˚C. Samples were frozen immediately at −20˚C and shipped frozen for analysis for I^- and Br^- (Symbio Laboratories, Eight Mile Plains, Queensland, Australia) and for $CHBr_3$ (Analytical Services Tasmania, New Town, Tasmania, Australia).

Immediately post-slaughter and dressing, hot standard carcass weight (HSCW; kg) was determined according to AUS-MEAT carcass standards [18]. After overnight chilling, the carcasses were ribbed, bloomed a minimum of 20 minutes, then evaluated for P8 fat depth (mm), eye muscle area (cm^2), ultimate pH, hump height (mm), rib fat depth (mm), marbling score, meat colour, and fat colour using Meat Standards Australia (MSA) grading and AUS-MEAT chiller assessment guidelines by MSA-accredited graders [18] [19]. Ossification was scored between 100 and 590 according to guidelines of the United States Department of Agriculture [20].

2.6. Consumer Sensory Evaluation

To ensure an even allocation for sensory evaluation, 4 left carcass sides were selected from each lot of 10, resulting in selection of 120 sides, 60 from the Control and 60 from the Asp-Oil groups. To randomize carcass selection, the first 4 carcasses in the grading order within a range of ossification, hump height and MSA marbling were prioritized from each lot number (10 head) and tagged with individual unique identification. The selected sensory population was indicative of the overall population within the study.

It was elected to sensory test the striploin HAM 2140 (*M.longissimus dorsi et cervicis*) and eye round HAM 2040 (*M.semitendinosus*) to provide two muscles of different muscle type and characteristics together with an expected large eating quality difference. The sides were boned, and the identified cuts were vacuum packed and chilled immediately before being transported to the Red Meat Innovation Center, Charles Sturt University (Wagga Wagga, New South Wales, Australia) for fabrication into consumer samples. Ageing comparisons were for 7, 14 and 28-days post-mortem for the striploin and 7 and 28-day for the eye round. Standard MSA consumer testing grill protocols [21] were utilized for all samples in the sensory study with all sensory work conducted by the Charles Sturt University sensory team and approved by the Charles Sturt University Human Ethics Committee (Approval H23526). Each of the 1200 consumers in the 20 sessions was served 7 samples. Five of the seven samples served were derived from the Control and Asp-Oil treatment groups with the remaining two providing linkage to other MSA tested product. Consumers recorded their quality judgements on individual scoring sheets for each of 7 samples. Scoring of the meat samples was achieved by placing a line across a 100 mm scale between the anchoring statements at either end of the scale, for each of the sensory variables (Not tender –

Extremely tender, Not juicy—extremely juicy, Dislike extremely—Like Extremely for Flavor and Overall scales) and then selecting one of 4 boxes labelled as unsatisfactory, good everyday, better than everyday and premium quality. Sensory scores were calculated as the average of the 10 consumers and clipped sensory scores created by removing the two highest and two lowest scores with the clipped value the mean of the central six.

2.7. Rumen Wall Examination

At slaughter, a subset of cleaned rumens was collected at random from Control (n = 30) and Asp-Oil (n = 30) groups and immediately received macroscopic gross pathology examinations for parameters of papillae color and shape, ventral sac development, and presence of lesions [22]. Briefly, papillae and ventral sac were evaluated visually for color, shape, and incidences of inflammation, lesions, and scaring as indicators of pathological differences between treatment groups. Color was subjectively scored on a scale between A-F; with A representing black/brown papillae and F yellow papillae. Papillae shape was subjectively scored on a scale between A-E; considering their length (long or short) and shape (thin, oval, or brittle). The ventral sac was scored on a 6-point scale with A being the least severe (no evidence of damage) and B-F representing different types of lesions (absence of papillae, scarring, erosions, hemorrhage, parakeratosis).

Tissue samples from the ventral sac of each rumen were collected for histological assessments. A semiquantitative assessment was conducted to evaluate the presence of ulcerations and fibrosis by measuring histological features, including acanthosis, sloughing of stratum corneum, dyskeratosis, keratinocytes with basophilic or mucinous cytoplasm, hydropic changes, and cytoplasmic vacuolation within the intraepithelial vesicles. Scoring of the histological features was based on the distribution of the lesion: grade 0 = absent; grade 1 = affecting < 20% of the papillae; grade 2 = affecting 20% - 50% of the papillae; grade 3 = affecting > 50% of the papillae. An additional semiquantitative method was conducted to evaluate the degree of parakeratosis over the entire histological section of each rumen tissue sample. The final parakeratotic score was calculated by multiplying the severity score [mild = 1, moderate = 2, or severe = 3] by the distribution score [<20% of papillae = 1, 20% - 50% of papillae = 2, or >50% of papillae = 3]. The final scores were then categorized by not present to mild [0 - 4.0], moderate [4.1 - 8.0], or severe [8.1 - 12] parakeratosis.

2.8. Methane and Hydrogen Emissions

Emissions monitoring for CH_4, carbon dioxide (CO_2), and hydrogen (H_2) were accomplished as described by Manafiazar *et al.* [23] and as visually depicted in Hristov *et al.* [24]. Briefly, four pens (2 Control, 2 Asp-Oil, in blocks 9 and 10) were fitted with GEM units (C-lock Inc., Rapid City, South Dakota, USA) to monitor CH_4 and hydrogen production (H_2 Production; g/day). GEM units were fixed for each pen and were not rotated during the experiment. The steers were

introduced to the GEM units and trained to visit and consume lure pellets that were presented in maximum 8 drops on individual visits with 30 seconds between drops, and 5 hours between visits. In this regime, the steers received maximum 32 drops each day consisting of 35 g pellets/drop. During each visit, while the steer has its head inside the canopy consuming the lure pellet, air was sampled from near the nose and mouth while the GEM unit also monitored airflow and ambient CH_4, CO_2 and H_2 flux during the visit period. The GEM units were calibrated weekly with standard Span and Zero gasses from C-lock Inc (Rapid City, South Dakota, USA). The CH_4 limit of detection (LOD) for these GEM units was a predicted CH_4 production value of up to 40 g CH_4/day (C-Lock, Rapid City, South Dakota, USA). We opted not to assign a default value of zero or treat it as censored data and impute values, rather, we retained all measurement values, treating the raw data as the best estimate of the true production, including those below the LOD. Two of the animals in the Asp-Oil treatment group exhibited markedly different behavior in terms of their CH_4 production levels than the other animals in the Asp-Oil group. The values recorded for these two were more in line with animals from the Control group. Rather than simply excluding these two outlying observations, we have computed two models as a way of determining the sensitivity of the results: one including the full data set and another that excluded the two unusual animals.

2.9. Statistical Analysis

All data processing and statistical analyses were conducted in R [25]. Data merging and manipulation, data visualizations and summary data were conducted using the Tidyverse suite of packages [26] and linear mixed models were fit with the lme4 package [27] with estimated marginal effects extracted using the emmeans package [28].

Dry matter intake was measured at the pen level and modelled as linear mixed models with treatment (Control or Asp-Oil) as the only fixed effect or allowing for an interaction between treatment and diet (d 0 - 21 or d 22 - 81) or liveweight period (d 0 - 40 or d 41 - 81) to obtain estimated marginal means for these subperiods. All models allowed for a random effect structure for pen within block (pairs of pens constituted a block). Liveweight and associated traits (ADWG, FCE, and cost of feed), and carcass measurements were analyzed at the pen level with a series of linear models where each included the treatment factor as a predictor.

Residue data for $CHBr_3$, and Br^- and I^- contained variable proportions of measurements below the LOD and were therefore censored. In the analysis we considered the sensitivity of the estimated mean to three ways of treating the LOD: max-mean where the residue values below the LOD were replaced by the LOD value (0.10 mg/kg for $CHBr_3$ and I^-; 5 mg/kg for Br^-); mid-mean where the residue values were replaced by half the LOD value (0.05 mg/kg for $CHBr_3$ and I^-; 2.5 mg/kg for Br^-); and min-mean where the residue values were replaced with zero values. The max-mean and min-mean show the range of possible outcomes for the mean

and the mid-mean represents a sensible middle ground.

Consumer sensory evaluations were analyzed with both clipped and raw consumer data were used for the analysis. Days ageing and carcass number were treated as characteristic variables. Treatment (Control or Asp-Oil), position within cut and days ageing were used as fixed variables with carcass number treated as the random intercept in the linear models. Sensory scores were calculated as the average of the 10 consumers and clipped sensory scores created by removing the two highest and two lowest scores with the clipped value the mean of the central six. The clipped scores are utilized for prediction modelling as they reduce the impact of outliers.

For the subset of animals with CH_4 and H_2 (g/day) production measurements, observations were grouped into 3-hour blocks of time aligning with diurnal cycles in production patterns given the feeding schedule. The fitted models allowed for a three-way interaction between time of day (a factor with eight 3-hour blocks), treatment (Control or Asp-Oil) and diet (d 0 - 21 or d 22 - 81). The animal identifier was included as a random intercept. When calculating the overall production, predictions for each treatment group were averaged over the eight levels of time of day.

3. Results

3.1. Feedlot Performance Characteristics

Effects on productivity and performance in feedlot steers induced by feeding Asp-Oil compared to the Control are presented in Table 2. The most compelling effect was a significant benefit to FCE of 7.4% ($P = 0.018$) over the final 40 days of the finisher diet of d 41 - 81, and of 5.6% ($P = 0.004$) for the full feedlot period inclusive of the transition steps of d 0 - 81. Contributing to the significant gains in FCE in the Asp-Oil steers was the significantly lower DMI of 3.3% ($P = 0.022$) and 4.1% ($P = 0.005$) in the finisher period of d 22 - 81 and final feeding period of d 41 - 81, respectively. Overall, for the full feedlot period of d 0 - 81, DMI of Asp-Oil steers tended to be lower than the Control by 2.6% ($P = 0.071$). In contrast, during the collective steps of the transition and adaptation period of d 0 - 21 there was no difference in DMI ($P = 0.884$).

The cost of feed and cost of liveweight gains are presented in Table 2. As a result of the improved FCE, the cost to feed the Asp-Oil steers during the finishing period of d 41 - 81 was reduced significantly by 4.6% ($P = 0.045$). There was no significant difference in feed costs while the steers were receiving the transition diets ($P = 0.669$). Overall, the 2.9% ($P = 0.133$) cost of feed reduction for the entire feedlot period was not significant, although may be functionally important in commercial feedlot settings. However, the cost of gain for the full feedlot period benefited significantly from a reduction of 4.3% ($P = 0.007$).

Unexpectedly and unrelated to the treatment, there were mortalities in three steers in their respective pens. Veterinary examination confirmed that inclusion of Asp-Oil did not contribute to the manifestation of symptoms causing mortality,

although coincidentally, the three were being offered Asp-Oil in their TMR. One steer succumbed to acidosis, another to bloat, and a third to a sustained unwitnessed and untreatable back injury likely incidentally inflicted by his conspecifics. During the duration of the study, there were no steers requiring residence in the dedicated hospital pen and no further incidents or health conditions observed.

Table 2. Animal feedlot performance least-squared means of short-fed *Bos taurus* steers fed a barley-based feedlot diet supplemented with canola oil (Control) or *Asparagopsis* bioactives stabilized in canola oil (Asp-Oil) delivering up to 35 mg bromoform/kg dietary DM, for an 81-day feeding period.

Parameter	Control	Asp-Oil	SE	*P*
DMI[1] (kg DM/head/d^{-1})				
Day 0 - 21	9.32	9.30	0.17	0.884
Day 22 - 81	11.69	11.31	0.15	0.022
Day 0 - 40	10.21	10.13	0.16	0.614
Day 41 - 81	11.91	11.42	0.16	0.005
Day 0 - 81	11.05	10.76	0.14	0.071
Liveweight (kg)				
Day 0	374	372	10.60	0.903
Day 40	482	483	12.02	0.953
Day 81	566	569	12.23	0.795
ADWG (kg/d)				
Day 0 - 40	2.72	2.77	0.09	0.571
Day 41 - 81	2.10	2.16	0.07	0.345
Day 0 - 81	2.38	2.43	0.05	0.229
FCE (ADWG/DMI)				
Day 0 - 40	0.267	0.275	0.01	0.262
Day 41 - 81	0.176	0.189	0.01	0.018
Day 0 - 81	0.215	0.227	0.00	0.004
Cost of feed ($AUD/head·d^{-1})				
Day 0 - 40	6.62	6.57	0.058	0.669
Day 41 - 81	7.67	7.32	0.086	0.045
Day 0 - 81	7.16	6.95	0.071	0.133
Cost of gain ($AUD/kg liveweight)				
Day 0 - 40	2.44	2.37	0.07	0.307
Day 41 - 81	3.77	3.54	0.09	0.022
Day 0 - 81	3.01	2.88	0.05	0.007

[1]Total intake, including feedlot ration, and GreenFeed Emissions Monitor attractant pellets.

Comparisons of cost of feed and cost of gain are specific to this study and

account for the current and relative feed pricing associated with the present feedlot operator. That said, reductions in feed costs translated to AUD $0.35 and $0.21 per head/d during the finisher liveweight measurement period of d 41 - 81 and over the duration of the study for d 0 - 81, respectively. Concomitantly, cost per kg of liveweight gain was reduced by AUD $0.23 and $0.13 during the finisher period for d 41 - 81 and the full feedlot period for d 0 - 81, respectively.

3.2. Carcass Performance and Residues

Carcass performance outcomes are presented in Table 3. Overall, there was negligible effect on carcass characteristics induced by feeding Asp-Oil. Compared to Control, the Asp-Oil steers P8 fat tended to be deeper by 6.1% ($P = 0.096$). Otherwise, there was no significant differences between Control and Asp-Oil in their HSCW, eye muscle area, ultimate pH, AUS-MEAT or MSA marbling scores, AUS-MEAT meat or fat color scores, ossification scores, or MSA index.

Table 3. Carcass grading least-squared means of short-fed *Bos taurus* steers fed a barley-based feedlot diet supplemented with canola oil (Control) or *Asparagopsis* bioactives stabilized in canola oil (Asp-Oil) delivering up to 35 mg bromoform/kg DMI for an 81-day feeding period.

Carcass Performance	Control	Asp-Oil	SE	*P*
Hot standard carcass weight (kg)	304	306	6.73	0.725
Dressing (%)	53.6	53.8	0.20	0.578
P8 fat (mm)	9.56	10.14	0.33	0.096
Eye muscle area (cm^2)	82.0	81.8	1.23	0.855
Ultimate pH	5.54	5.56	0.02	0.202
Hump height (mm)	59.9	61.4	0.82	0.079
Rib fat (mm)	6.41	6.33	0.37	0.845
Ossification score	140	139	1.54	0.834
AUS-MEAT marbling score	0.70	0.65	0.06	0.463
MSA marbling score	294	290	6.58	0.627
AUS-MEAT meat color	3.71	3.81	0.07	0.154
AUS-MEAT fat color	0.42	0.33	0.05	0.103

Residue analysis results of meat and edible offal are presented in Table 4. When all analyses were below the LOD (n = 0) then not detected (ND) was reported. When all analyses were above LOD (n = 10) then a single mean of residue (mg/kg) was reported. When a variable number of results were both above and below LOD (n = 1 - 10) then a maximum and minimum residue was reported based on the censored result. No residues of Asp-Oil $CHBr_3$ were detected in any of the meat, liver, and kidney samples of either Control or Asp-Oil treatment groups. However, trace levels of Br^- and I^- were detected in both groups with incidences of ND and measurements above the LOD for the same analyte and tissue type. Trace Br^-

was detected in all samples of kidney from both the Control and Asp-Oil steers averaging 11.7 and 18 mg Br^-/kg, respectively. The Control liver and meat samples were all under the LOD for Br^- (5 mg/kg). Trace Br^- was detected in all liver samples of Asp-Oil steers with mean Br^- of 7.5 mg/kg. Meat from the Asp-Oil steers had trace (6 of 10) and ND (4 of 10) Br^- results in a range bridging the LOD of 3.4 - 5.4 mg Br^-/kg of meat.

Table 4. Bromoform, bromide and iodide maximum and minimum means for muscle, liver, and kidney samples taken from short-fed *Bos taurus* steers fed a barley-based feedlot diet supplemented with canola oil (Control) or *Asparagopsis* bioactives stabilized in canola oil (Asp-Oil) delivering up to 35 mg bromoform/kg DMI for an 81-day feeding period.

	Control (mg/kg)			Asp-Oil (mg/kg)		
Bromoform[1]	max.	min.	n[2]	max.	min.	n[2]
Meat	ND[3]		0	ND		0
Liver	ND		0	ND		0
Kidney	ND		0	ND		0
Bromide[1]						
Meat	ND		0	5.4	3.4	6
Liver	ND		0	7.5		10
Kidney	11.7		10	18.0		10
Iodide[1]						
Meat	ND		0	ND		0
Liver	0.10	0.03	3	0.10	0.09	9
Kidney	0.15		10	0.17		10

[1]LOD for bromoform and iodide was 0.10 mg/kg; bromide was 5 mg/kg; [2]n = number of samples at or above the LOD for each treatment group; [3]ND = all measurements were non detects / below the LOD.

Trace I^- was detected in all samples of kidney from both the Control and Asp-Oil steers at 0.15 and 0.17 mg I^- /kg, respectively. The Control steers presented trace liver I^- as well as ND resulting in a range bridging the LOD of 0.03 - 0.10 mg I^-/kg of liver. Likewise, The Asp-Oil steers presented a range bridging the LOD of 0.09 - 0.10 mg I^-/kg of liver. No residues of I^- were detected in meat from the Control and Asp-Oil steers.

3.3. Consumer Sensory Evaluation

The consumer sensory evaluation scores are presented in Table 5. Meat from the Asp-Oil steers was considered better overall but not statistically significant ($P > 0.05$). Based on 1,200 consumer responses, in the striploin cut meat eating quality comparisons there was approximately a 1.8 point difference in Tenderness, 0.9 point difference in Juiciness, 1.0 point difference in Flavor and 0.9 point difference

in Overall Liking between the Control and Asp-Oil striploin samples, in favor of the Asp-Oil treatment group. This equated to an overall Clipped Meat Quality (CMQ4) score difference of 1.1 points, which was not significant. The differences observed in the eye round cut meat eating quality comparisons there was less than a 0.5 point difference in Tenderness, 0.6 point difference in Juiciness, no difference in Flavor, 0.4 point difference in Overall liking, and again in favor of the Asp-Oil treatment group. This equated to an overall CMQ4 score difference of 0.3 point, also not significant.

Table 5. Summary statistics for clipped scores for tenderness, juiciness, flavor, overall liking and total meat quality, and satisfaction score by cut (striploin and eye round) from short-fed *Bos taurus* steers fed a barley-based feedlot diet supplemented with canola oil (Control) or *Asparagopsis* bioactives stabilized in canola oil (Asp-Oil) delivering up to 35 mg bromoform/kg DMI for an 81-day feeding period.

	Striploin sample		Eye round sample	
	Control	Asp-Oil	Control	Asp-Oil
	(N = 180)	(N = 180)	(N = 180)	(N = 180)
Tenderness				
Mean (SD)	53.9 (12.4)	55.7 (11.4)	31.9 (12.2)	32.4 (12.3)
Median [Min, Max]	54.2 [25.0, 83.8]	56.3 [20.2, 84.5]	30.8 [6.83, 68.7]	31.9 [7.00, 61.8]
Juiciness				
Mean (SD)	49.7 (11.9)	50.6 (11.7)	36.4 (11.8)	37.0 (10.9)
Median [Min, Max]	49.5 [16.2, 82.0]	51.3 [22.8, 83.7]	35.1 [10.0, 70.0]	37.3 [12.5, 63.7]
Flavor				
Mean (SD)	55.3 (10.0)	56.3 (9.34)	42.0 (11.0)	42.0 (10.1)
Median [Min, Max]	55.2 [24.2, 84.0]	57.0 [35.0, 82.2]	42.0 [20.3, 79.2]	42.9 [18.3, 60.5]
Overall Liking				
Mean (SD)	54.2 (10.9)	55.1 (9.96)	38.0 (11.9)	38.4 (10.9)
Median [Min, Max]	53.5 [25.2, 81.5]	55.2 [30.0, 82.2]	36.8 [13.3, 81.0]	38.9 [10.8, 61.3]
CMQ4				
Mean (SD)	53.9 (10.2)	55.0 (9.50)	37.5 (10.8)	37.8 (10.1)
Median [Min, Max]	53.8 [24.9, 79.1]	55.3 [31.5, 82.5]	36.8 [16.2, 74.2]	39.2 [13.2, 59.9]
Satisfaction				
Mean (SD)	3.12 (0.351)	3.17 (0.376)	2.61 (0.395)	2.62 (0.354)
Median [Min, Max]	3.17 [2.17, 4.00]	3.17 [2.17, 4.50]	2.50 [2.00, 4.17]	2.55 [2.00, 3.33]

An estimated marginal mean plot presenting the clipped means is presented to visualize Tenderness, Juiciness, Flavor, Overall Liking, and MQ4 scores (**Figure 1**). It is evident that the group treated with Asp-Oil had benefited eating quality results

compared to the Control, with the visual improvement found across all sensory variables, the largest being Tenderness (1.3 points), although non-significant.

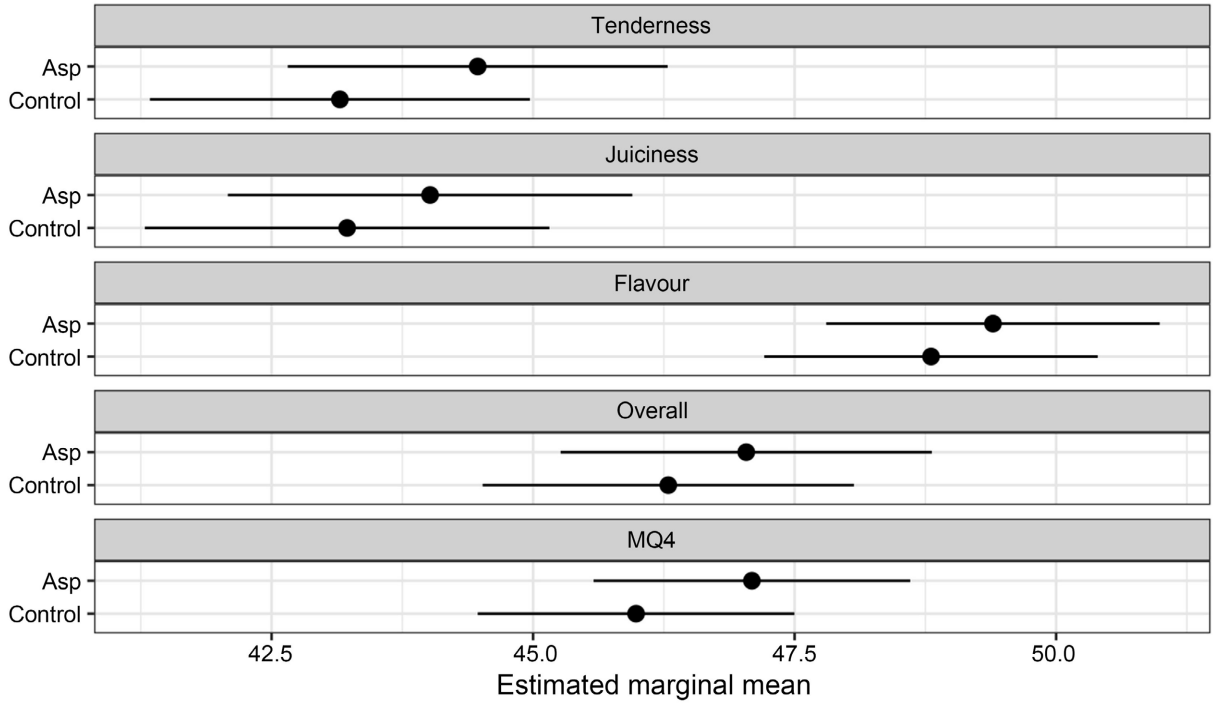

Figure 1. Estimated clipped marginal means plot for dependent variable of Tenderness, Juiciness, Flavor, Overall liking, and MQ4 by treatment for short-fed *Bos taurus* cattle fed a barley-based feedlot diet supplemented with canola oil (Control) or *Asparagopsis* canola oil (Asp-Oil) delivering 35 mg bromoform /kg DMI for an 81-day feeding period.

3.4. Rumen Wall Examination

Table 6 shows the scoring distribution and characterization of the subset of rumens evaluated in the rumen wall visual examinations for both Control (n = 30) and Asp-Oil (n = 30) groups. Most of the rumens scored either A, B, or C in each of the rumen health assessment factors of papillae color, papillae shape, and ventral sac health which indicates that overall, both groups demonstrated normal rumen health and development typical of feedlot style diets. Both Control and Asp-Oil groups demonstrated appreciable variation in papillae color, with steers achieving scores between D - F in 37% and 13% of rumens, respectively. Correspondingly, appreciable variations in scores between C - E were observed for papillae shape in 17% and 23% of rumens of Control and Asp-Oil steers, respectively, and for ventral sac health in 13% and 33% of rumens of Control and Asp-Oil steers, respectively. Incidence of ventral sac macroscopic parakeratosis achieving a score of F was observed in 3.3% and 37% in rumens of Control and Asp-Oil steers, respectively. Star-shaped scars occurred on the pillars in 3.3% and 10% of the rumens of Control and Asp-Oil steers, respectively. Given the chronicity of the scars, it is likely the lesions originated and resolved before the commencement of the study. None of the rumens from either Control or Asp-Oil were observed to have red or bloody areas.

Table 6. Characterization of papillae color and shape, and ventral sac in short-fed *Bos taurus* cattle fed a barley-based feedlot diet supplemented with canola oil (Control) or *Asparagopsis* canola oil (Asp-Oil) delivering up to 35 mg bromoform /kg DMI for an 81-day feeding period.

		Group 1		Group 2		Group 3		Group 4		Group 5		Overall	
	Papillae Color	Control	Asp-Oil	Control	Asp-Oil	Control	Asp-Oil	Control	Asp-Oil	Control	Asp-Oil	Control	Asp-Oil
A	Black/Brown	0	3	0	2	1	2	0	5	0	2	1	14
B	Grey/Brown	4	3	1	2	0	1	4	1	5	2	14	9
C	Grey/Brown small area with pink tips	0	1	3	0	2	2	1	1	0	2	6	6
D	Grey/Brown large area with pink tips	0	0	0	2	0	0	0	0	0	1	0	3
E	Pink	2	0	0	0	0	0	0	0	0	0	2	0
F	Yellow	2	0	2	0	3	1	1	0	1	0	9	1
	Papillae Shape												
A	Long + Thin	6	2	5	6	6	4	5	6	5	6	27	24
B	Long + Oval	2	3	3	5	0	2	2	2	1	0	8	12
C	Short + Thin	2	0	0	1	0	1	1	2	2	2	5	6
D	Short + Oval	0	0	0	0	0	1	0	0	0	0	0	1
E	Short + Brittle	0	0	0	0	0	0	0	0	0	0	0	0
	Ventral Sac												
A	No evidence of any damage	6	5	4	4	6	3	6	6	4	4	26	22
B	Small areas bare of papillae	0	1	2	1	0	1	0	0	0	1	2	4
C	Large areas bare of papillae	2	2	0	1	0	1	0	0	1	0	3	4
D	Small areas of excoriation/scaring	0	0	0	2	0	2	0	0	1	2	1	6
E	Red/Bloody areas	0	0	0	0	0	0	0	0	0	0	0	0
F	Parakeratosis	0	1	0	1	1	2	0	5	0	2	1	11

In the histological examinations, microscopic parakeratosis was prevalent in rumens of both Control and Asp-Oil steers and presented with variable severity and distribution of occurrences. Table 7 presents the scoring results for the severity of acanthosis, sloughing of the stratum corneum, dyskeratosis, basophilic and mucinous cytoplasm, hydropic changes, and cytoplasmic vacuolation within intraepithelial vesicles. Both Control and Asp-Oil treatment groups experienced, to some degree, changes within the ventral sac of the rumen wall and scoring differences between the two treatment groups were minimal. The presence of focal or multifocal small aggregates of lymphocytes, plasma cells and neutrophils in the submucosa are prevalent in both groups, with the exception of cases with a severe acute inflammatory reaction associated with microabscesses; necrosis of the tips of the papillae or ulceration of the pillars. Microabscesses have been observed in 33% and 27% of rumens in Control and Asp-Oil steers, respectively. Microscopic parakeratosis was prevalent in rumens of both Control and Asp-Oil steers and

presented with variable severity and distribution of occurrences. Parakeratosis was classified in the rumens of the Control and Asp-Oil steers as: not-present to mild in 50% and 27% of occurrences; mild to moderate in 46% and 53% of occurrences; and moderate to severe in 3.3% and 17% of occurrences; respectively. Combined average parakeratosis scores for Control and Asp-Oil treatment groups was 4.6 ± 1.4 and 6.2 ± 1.7, respectively, and both would be categorized as moderate parakeratosis.

Table 7. Semiquantitative histological assessment of rumen ventral sac tissue samples collected from short-fed *Bos taurus* cattle fed a barley-based feedlot diet supplemented with canola oil (Control) or *Asparagopsis* canola oil (Asp-Oil) delivering 35 mg bromoform /kg DMI for an 81-day feeding period.

Feature	Grade	Control (%)	Asp-Oil (%)
Acanthosis	0	0.00	0.00
	1	3.33	10.7
	2	66.7	21.4
	3	30.0	67.9
Sloughing of the stratum corneum	0	56.7	51.7
	1	43.3	31.0
	2	0.00	17.2
	3	0.00	0.0
Dyskeratosis	0	13.3	6.67
	1	43.3	48.3
	2	33.3	31.0
	3	10.0	13.8
Basophilic/mucinous cytoplasm	0	33.3	17.2
	1	33.3	51.7
	2	30.0	31.0
	3	3.33	0.00
Hydropic changes	0	3.33	6.67
	1	76.7	69.0
	2	16.7	20.7
	3	3.33	3.33
Intraepithelial vesicles	0	73.3	69.0
	1	23.3	27.6
	2	3.33	3.33
	3	0.00	0.00

3.5. Methane and Hydrogen Emissions

The mean number of visits which resulted in a CH_4 recording from the GEM units

ranged from 11.4 - 16.2 per pen for all 4 monitored pens, with a total of 4528 measurements (Figure 2). There were 3 days with missing or very low readings influenced by flooding in all the pens containing the GEMs. Confoundingly, the Control and Asp-Oil treatment steers were below the 40 g CH_4/d LOD of the GEM units for 15% and 67%, respectively. These below LOD measurements, for the Asp-Oil group particularly, confounded characterization of substantive CH_4 mitigation and contributed to underestimation of anti methanogenic efficacy. The individual data points are presented in a cloud diagram in Figure 2, illustrating the density of measurements below 40 g CH_4/d.

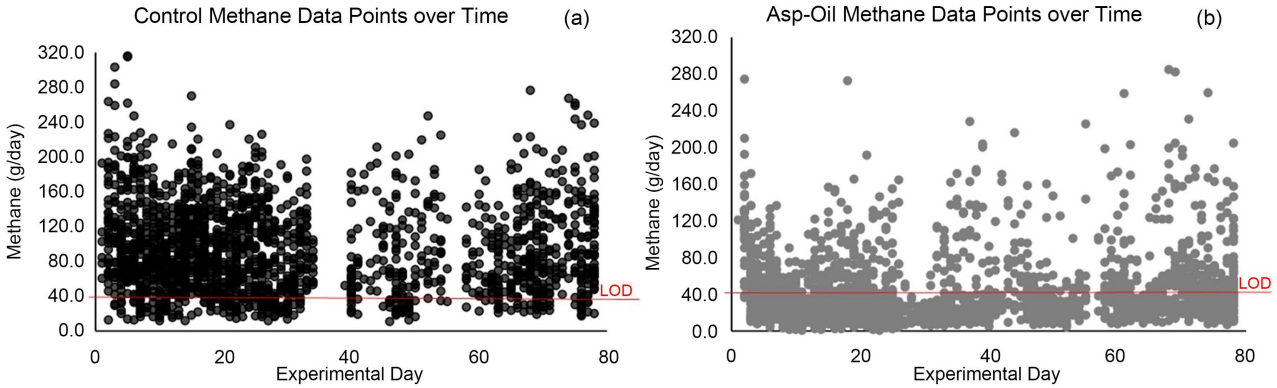

Figure 2. Individual methane (CH_4) measurements for all steers for the duration of the study (d 0 - 81). The plots present measurements relative to the 40 g CH_4/d Limit of Detection (LOD; red line) of the GEM used in this study. Plot (a) represents the Control group with 15% of measurements below LOD and Plot (b) represents the Asp-Oil treatment group with 67% of measurements below the LOD.

The CH_4 emissions results as measured using the GEM units for all periods of the study are presented in Table 8. Based on the preliminary study [3] respiration chamber data, confidence is high that LOD values fall in the range of 0 - 40 g/d and are skewed substantively toward zero. However, the GEM data is presented as acquired and subsequently, we refer to Cowley *et al.* [3] for the >98% upper bound for the expected range of emissions reduction. Collectively, the CH_4 production assembled from the GEM units and Cowley *et al.* [3] provide the range of CH_4 emissions reduction of Angus-Shorthorn cattle on a feedlot finisher ration of 58% - 98%.

The H_2 emissions results as assembled using the GEM units for all periods of the study are presented in Table 8. Hydrogen emissions were relatively consistent throughout the full feedlot period at 0.7 - 0.8 g/day in the Control group. The Asp-Oil group's H_2 emissions also remained consistent, however, was approximately seven times higher at 5.3 - 5.4 g/d (Table 8, Figure 3). The H_2 emissions inversely reflected the CH_4 emissions and high levels of CH_4 emissions from Control steers were concomitant with low levels of H_2, and the low CH_4 emissions from the Asp-Oil steers were concomitant with high H_2 emissions. Subsequently, the Asp-Oil steers had significant increases in H_2 emissions of 657%, 575%, 671% ($P < 0.001$) in the transition (d 0 - 21), finisher (d 22 - 81), and full feedlot periods (d 0 - 81),

respectively.

Table 8. Mean methane (CH_4: g/day) production and hydrogen (H_2: g/day) production for short-fed *Bos taurus* steers fed a barley-based feedlot diet supplemented with canola oil (Control) or *Asparagopsis* canola oil (Asp-Oil) delivering 35 mg bromoform /kg DMI for an 81-day feeding period.

	Control	Asp-Oil (inc.)[1]	Asp-Oil (exc.)[2]	*P*[3]
Methane production, g CH_4/day				
Day 0 - 21	94.0	37.1	35.6	<0.001
Day 22 - 81	98.3	41.7	33.6	<0.001
Day 0 - 81	96.2	39.4	34.6	<0.001
Hydrogen production, g H_2/day				
Day 0 - 21	0.65	5.32	5.27	<0.001
Day 22 - 81	0.84	5.45	5.62	<0.001
Day 0 - 81	0.74	5.38	5.45	<0.001

[1]Asp-Oil means including all animals, [2]Asp-Oil means excluding two animal outliers; [3]*P*-values do not change with inclusion and exclusion of outliers.

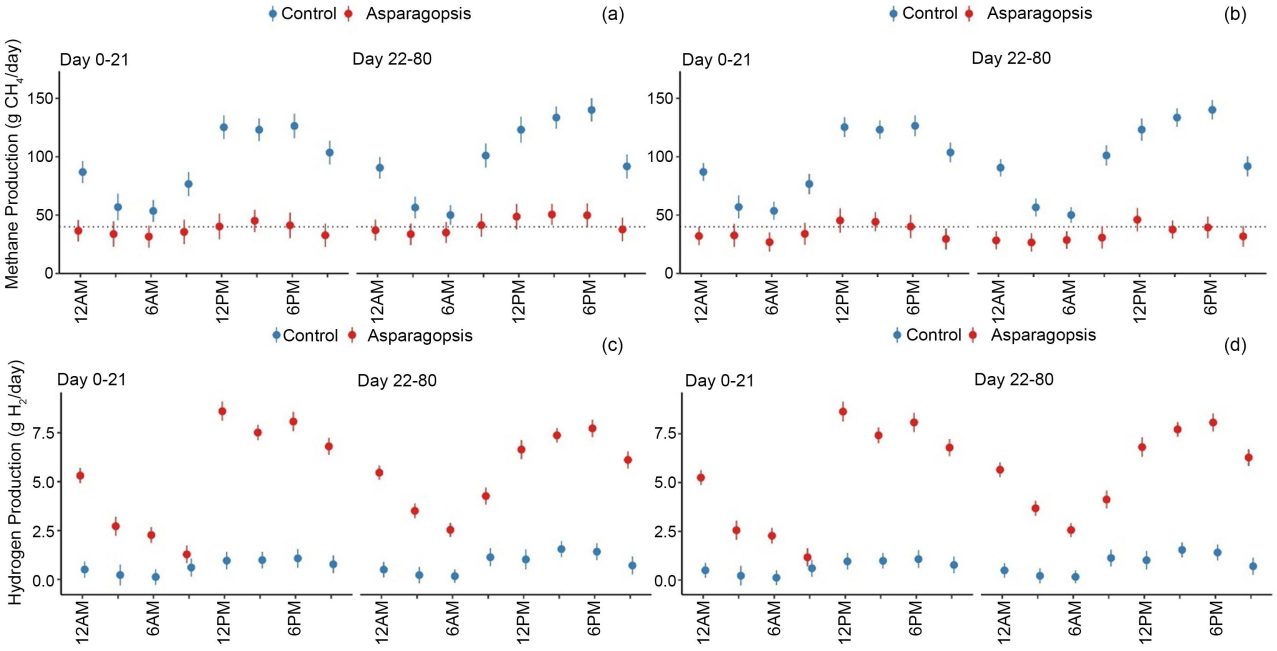

Figure 3. Predicted methane (CH_4) and hydrogen (H_2) production means for the linear mixed model diet (transitions and finisher), hour of day, and treatment as fixed effects and allowing for a random intercept for animal ID. Plots (a) and (c) include the two outlier steers in the Asp-Oil treatment group, and plots (b) and (d) excludes them. The CH_4 Limit of Detection of 40 g/d is indicated as the horizontal dotted line in plots (a) and (b). Each tick mark on the x-axis (each point in the plot) aggregates over the following 3 hour time block (ex. 12 AM tick represents observations from midnight to 3 AM).

All but two steers in the monitored Asp-Oil pens averaged CH_4 production at or under the GEM's LOD. In a further complication, these two steers often exceeded the CH_4 production of the Control mean CH_4 production. Although they

are outliers, the lack of definitive insight on why they did not show reductions compared to their Asp-Oil cohorts makes it inappropriate to clip them. Therefore, two scenarios have been presented where they are retained as part of their cohort and are removed as outliers (**Table 8**, **Figure 3**).

Compared to the Control, on average, during the finisher period when the steers were on full inclusion of Asp-Oil (d 22 - 81), there was significant inhibition of CH_4 production (g/d) induced by Asp-Oil of 57.6% ($P < 0.001$) inclusive of all steers, and 65.9% ($P < 0.001$) with the two outliers excluded (**Table 8**). The GEM estimates of significant CH_4 reductions during the full duration of the study (d 0 - 81), both inclusive and exclusive of the outliers, for production (g CH_4/d) were 59.0% and 64.1% ($P < 0.001$), respectively. Furthermore, CH_4 reductions induced by Asp-Oil were always significant during the study ($P < 0.001$).

The diurnal CH_4 and H_2 emissions, both including and excluding the CH_4 emissions outliers, are presented for the full feedlot period (d 0 - 81) in **Figure 3**. Across all hours of the day, it is illustrated that the Control animals produced significantly more CH_4 and in the early morning hours, the difference is reduced. Emissions ebb and flow in a consistent pattern throughout the day, independent of diet change across the periods of the study. The pattern is more pronounced for the Control whilst the Asp-Oil steers are comparatively stable due to consistent CH_4 production below the LOD. The Control group emissions rise rapidly to peak a few hours after morning feed offering and drop dramatically overnight to the early morning hours [29]. After the diet transition (d 0 - 21) and full Asp-Oil inclusion was achieved, the mean CH_4 production consistently falls below the LOD for both scenarios. However, CH_4 is consistently higher and more variable when retaining the outliers (**Figure 3(a)**) compared to removing them (**Figure 3(b)**). Plot (a) compared to (b) illustrates a marked difference in CH_4 emissions in the Finisher period (d 22 - 81) when comparing retaining the outliers (a) versus removing them (b). Conversely, plots (c) compared to (d) in **Figure 3** illustrates that H_2 emissions remain unchanged and are not affected by inclusion or exclusion of the two CH_4 emitter outlier steers.

4. Discussion

4.1. Effects on Feedlot and Carcass Performance and Residues

This study provides confirmation of the beneficial impact of feeding *Asparagopsis* products on beef cattle productivity and CH_4 abatement in a commercial feedlot without incidental effect on meat quality, animal health, and food safety. The trend of lower DMI ($P = 0.071$) in Asp-Oil steers compared to Control steers concomitant with no change in ADWG resulted in a significant improvement in FCE ($P = 0.004$).

Improvements in FCE were accordingly ascribed within multiple perspectives contingent with experimental design. As is typical for adaptation to the feedlot finisher ration, a four-step transition was used (**Table 1**). The transition steps provided opportunity to introduce the Asp-Oil into the diet gradually such that when

the steers started on the finisher diet they correspondingly started their full inclusion of Asp-Oil. Consequently, at less than full and optimal inclusion, the CH_4 mitigation efficacy may not yet be stabilized, and therefore the rumen would not be acclimatized to low CH_4 and higher H_2 conditions. It has been reported that Asp-Meal induces greater ADWG and FCE improvements during the finisher period compared to the higher forage transition diets [2]. Kinley *et al.* [1] reported that peak benefits were achieved later in the finisher period, presumably after the rumen ecosystem has acclimatized fully to the diet and changes in rumen gas composition. In the latter study the steers were not introduced to Asp-Meal until the start of the finisher diet. More intensive measurements in commercial production are warranted to characterize productivity relative to the transition timeline, however, such activity could be counterproductive with feedlot cattle that may respond negatively to the disruption. In the present study, no liveweight measurement was collected to isolate FCE for the transition period. There was a numerical improvement in FCE (P = 0.262) of 3.0% for the collective transition and first third of the finisher periods (d 0 - 40). Subsequently, there were significant improvements induced by Asp-Oil compared to the Control in FCE of 7.4% (P = 0.018) for the final two-thirds of the finisher period (d 41 - 81) which was extensive enough to influence the full duration of the study (d 0 - 81) which was 5.6% (P = 0.004) greater than the Control group.

Some studies exploring the benefits of feeding *Asparagopsis* to feedlot beef cattle have reported considerable productivity gains. However, gains could not be confirmed due to a lack of statistical power [1] [2]. Even small improvements in FCE are important to large scale commercial adoption of *Asparagopsis* products in the beef, sheep, and dairy industries. Confirmation of FCE benefits with no detriment to food or animal is expected to provide confidence for livestock producers, who are increasingly under pressure due to rising feed prices and scrutinized as consumer sentiment intensifies over the contribution of livestock farming on global greenhouse gas inventories. Reduction in cost of feed and liveweight gains coupled with extensive reductions in CH_4 emissions has potential to drive adoption and improve the image of the wider beef, sheep, and dairy sector. Furthermore, climate conscious consumers may increase their interest in food products with a foundation of tangible emissions reductions as they are increasingly aware of industrial greenwashing [30].

The 4.3% reduction in cost of weight gain demonstrated in the present study has been previously reported on a feedlot diet typical of the USA market. Roque *et al.* [2] reported significant gains in FCE and subsequent, but nonsignificant, 4.9% improvement in cost of liveweight gain in their finisher diet. The present study demonstrates that inclusion of Asp-Oil would result in considerable cost of production savings compared to feedlot steers not receiving Asp-Oil.

Decline in DMI in ruminants supplemented with *Asparagopsis* under intensive experimental conditions with a low number of animal replicates has been reported previously [2] [4] [12] [31]. Palatability has previously been speculated as a cause

of reduced DMI [4]. However, Asp-Oil included in a grain concentrate supplement twice per day at milking to dairy cows induced only marginal reduction in supplement intake and no significant change in total DMI [5] [32]. The researchers reported that reduction in forage intake occurred in only a small proportion of cows during the Asp-Oil ramp-up period. The concentrated nature of Asp-Oil pulse delivery requires the daily allotment to be consumed in two offerings as opposed to TMR distribution through the entire daily DMI. Only marginal reduction in the supplement intake indicates that impaired palatability was not an issue for Asp-Oil inclusion in a pulse-delivery supplement [5] [32] and would be even less likely to affect DMI in a TMR when offered within the recommended REIL [3].

It has been theorized that increased H_2 pressure in the rumen may interfere with efficient rumen function and subsequently impair DMI and ruminant productivity [33] [34]. However, studies are consistently contesting that theory, including the present study, and demonstrating that increased H_2 pressure as a result of significant CH_4 inhibition is not detrimental to ruminant productivity, and subsequently reporting improvements in ADWG and FCE [1] [2] [35] [36]. Martinez-Fernandez *et al.* [36] reported significant CH_4 inhibition with increased ADWG and that the concomitant H_2 expected to be emitted was not fully achieved, thus indicating redirection of metabolic hydrogen [H] to beneficial end products. Furthermore, redirection of [H] and significant increase in eructated H_2 in these studies suggest that it has a limited ability to accumulate in the rumen [36] [37]. Additionally, Martinez-Fernandez *et al.* [38] and Romero *et al.* [39] advocate that emission of H_2 is a loss of feed energy that has potential to be partially redirected and H_2 pressure reduced to improve rumen fermentation and further enhance productivity gains. The authors suggest this may be achieved by addition of some form of electron acceptors coinciding with CH_4 mitigation, with phloroglucinol being one effective example. Interestingly, phloroglucinol is a common phenolic derivative of brown seaweeds [40] and has been isolated from the phytochemicals of red seaweed [41]. Exploration of phloroglucinol in *Asparagopsis* and potentially functional blends with brown seaweeds, studying effects on ruminal H_2 and rumen fermentation, is warranted.

The $CHBr_3$, Br^- and I^- residues in muscle, kidney and liver conform with previous findings that meat and offal from cattle fed Asp-Oil of up to 51 mg $CHBr_3$/kg DMI was safe for human consumption [3]. **Table 4** reports that $CHBr_3$ was not detected in any sample of muscle, liver or kidney, and agrees with previous reports [1] [2] [42].

In meat, I^- was not detected and although trace levels were detected in liver and kidney they were well within safe levels of consumption. Daily tolerable upper limits on a mg/day basis for iodine consumption are as follows: under age 3 (0.2); age 4 - 8 (0.3); age 9 - 13 (0.6); age 14 - 18 (0.9); age 19+ (1.1) [43]. The maximum mean for iodide was 0.17 mg/kg in kidney and 0.10 mg/kg in liver from the Asp-Oil group. A child under the age of 3 would have to consume more than 1.2 kg of

kidney or 2 kg of liver daily to exceed the tolerable upper limits. An adult over the age of 19 would have to consume more than 6.5 kg of kidney or 11 kg of liver daily to exceed the tolerable upper limits.

Meat, kidney, and liver Br^- concentrations were marginally higher in Asp-Oil carcasses than in Control carcasses but were well within recommended safe levels for human consumption. Daily tolerable upper limit for Br^- consumption is 1 mg/kg BW [44] with a conservative no observed effect level (NOEL) of 4 mg/kg BW [45]. The maximum mean for bromide was 18 mg/kg in kidney, 7.5 mg/kg in liver and 5.4 mg/kg in meat for the Asp-Oil group. A child between the ages of 1 - 3 (average estimate of 13 kg), would have to consume more than 0.7 kg of kidney, 1.7 kg of liver, or 2.4 kg of meat every day to exceed the tolerable upper limits. To exceed the NOEL levels, the child would have to consume more than 2.9 kg of kidney, 6.9 kg of liver, or 9.6 kg of meat daily. An adult (average estimate of 70 kg) would have to consume more than 3.9 kg of kidney, 9.3 kg of liver, or 13 kg of meat daily to exceed the tolerable upper limits. To exceed the NOEL levels, the adult would have to consume more than 15.6 kg of kidney, 37.3 kg of liver, or 51.9 kg of meat daily.

4.2. Effects on Consumer Sensory Evaluations

This study confirmed through sensory evaluation across 1200 consumers that there was no significant effect on meat eating quality due to Asp-Oil supplementation in the TMR of feedlot cattle. Although not significant, there was functional improvement in all clipped sensory variables of the evaluation, particularly Tenderness and the collective of the traits expressed as MQ4 (**Table 5**; **Figure 1**). Days ageing and positional effects within the striploin and eye round cuts were not adversely affected in the Asp-Oil group with overall results aligning with MSA model industry expectations. Overall, for both Control and Asp-Oil steers, eating quality results were slightly lower than expected for cattle of the quality used for this study. A potential contributor may be the use of a hormone growth promotant (HGP) in all the steers.

There is scope to reduce CH_4 emissions by using Asp-Oil as a feed additive, while improving productivity without compromising meat-eating quality. This provides industry with a potential tool for mitigating carbon that will not have adverse effects on grading results or overall eating quality. This data will contribute to providing industry with the confidence that the MSA model can accurately predict eating quality results of cattle receiving Asp-Oil inclusion in a feedlot TMR. Furthermore, confidence in producers that feed inclusion of Asp-Oil and *Asparagopsis* products in general will not negatively affect their high-quality meat products will be important in driving commercial adoption.

4.3. Effects on Rumen Wall

Rumen wall parakeratosis is thought to be caused by drops in pH and a rapid accumulation of volatile fatty acids (VFAs) in the rumen and is most common in

animals fed a high-concentrate ration or heat-treated alfalfa pellets [46]. The presence in both Control and Asp-Oil groups of focal or multifocal small aggregates of lymphocytes, plasma cells and neutrophils in the submucosa can be interpreted as an incidental finding since it was not associated with any major changes in the mucosal epithelium. Microabscesses were more prevalent in the Control (33.3%) compared to Asp-Oil (26.7%), which are a result of a more severe progression of parakeratosis. Similar parakeratosis lesions that could not be confirmed as related to dietary *Asparagopsis* have been described in 12 sheep fed a pelleted cereal straw, lupin seed, and mixed grain-based diet and top dressed with supplemental lupin seed and *Asparagopsis* [42]. Ruminants fed a high grain diet typical of feedlot cattle commonly present rumen parakeratosis that may reduce normal absorption mechanisms for VFAs. However, this theory has not been scientifically supported [46] and the current study directly contradicts this with improved productivity shown in the Asp-Oil steers, although they had a moderately higher incidence of rumen hyperkeratosis and parakeratosis. Magrin *et al.* [47] reported an extensive beef feedlot study where the post-mortem inspection of 2116 rumens at the slaughterhouse revealed the presence of hyperkeratosis and or parakeratosis in 58% of the animals. That said, the cattle in the present study were within the scope of common occurrence of rumen abnormalities in the general population of feedlot cattle [47].

4.4. Effects on Methane and Hydrogen Emissions

Methane emissions represent a loss of feed energy from the rumen and inhibition of CH_4 synthesis has been hypothesized to have potential to redirect energy to more advantageous hydrogen sinks, such as the VFA propionate, which is a precursor of glucose in the ruminant liver [37]. Morgavi *et al.* [13] prepared a meta-analysis of experiments inhibiting methanogenesis, suggesting no consistent relationship between inhibition of CH_4 production and growth efficiency. Unfortunately, the authors supported the claim using studies with limited CH_4 inhibition with a low CH_4 reduction of 30%. Using only a 30% reduction diluted the opportunity to elucidate the association, considering it is almost threefold lower than those reporting significant performance benefits in beef production [1] [2]. Likewise, the caveat expressed in their report was that studies to date were not sufficiently powered and using sufficient animals could elucidate the association between CH_4 inhibition and performance gains [13].

The present study demonstrates the relationship between enteric CH_4 mitigation and productivity gains through demonstration of 58% - 98% CH_4 inhibition (**Figure 4**) as well as significantly improving FCE. This study reports on the commercial scale demonstration of the effective Asp-Oil inclusion level ascertained in the preliminary REIL study [3]. In that work using the gold-standard open-circuit respiration chambers as the emissions monitoring tool it was confirmed that the inclusion of Asp-Oil to deliver 34 - 51 mg $CHBr_3$/kg DMI in the analogous tempered barley-based feedlot diet resulted in average 89 - 96% reduction in CH_4

production throughout the 77-day feedlot period. Inhibition of CH_4 production and yield in the isolated finisher diet period was >98% [3]. For functionality, respiration chambers were swapped for GEM units in the commercial feedlot environment. Subsequently, inclusion of Asp-Oil to deliver 35 mg $CHBr_3$/kg DM, resulted in lower inhibition efficacy measured compared to the respiration chamber study, although the majority of CH_4 was inhibited. In this study scenario, the respiration chambers were able to accurately measure trace amounts of CH_4 and provide a better estimate of the true CH_4 reduction [1] [3] [42].

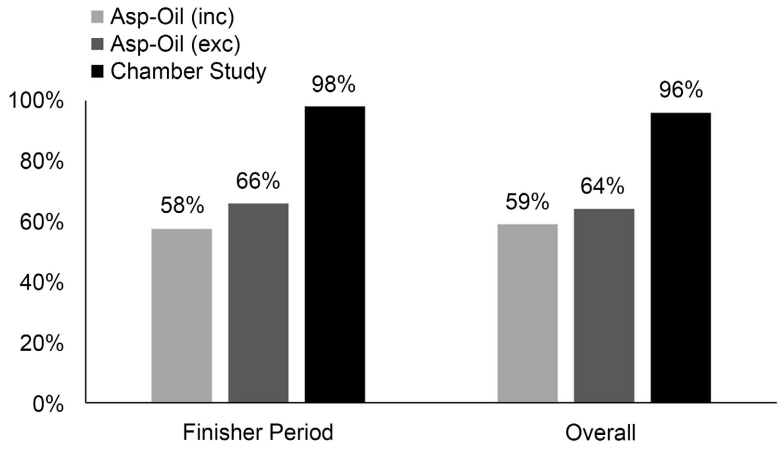

Figure 4. Comparison of CH_4 production (g/day) mitigation induced by Asp-Oil in feedlot cattle of similar breed, consuming similar diet with the same inclusion level of Asp-Oil (35 mg $CHBr_3$/kg DMI) and accounting for the preliminary experiment measurements using respiration chambers ([3]; chamber study) and GEM units (Asp-Oil inc & Asp-Oil exc). Asp-Oil (inc) represents efficacy with all animals included whereas Asp-Oil (exc) represents efficacy with the two outliers removed.

The GEM technology has the capability for deployment in commercial settings to provide for emissions monitoring which would not be possible otherwise without disturbing the natural behavior of cattle in the feedlot. A key disadvantage was that CH_4 inhibition induced by Asp-Oil exceeded the functional LOD of 40 g CH_4/day of the GEM units with most measurements. Furthermore, GEM units apply a voluntary spot-check sampling method compared to continuous monitoring in respiration chambers.

It has long been known that CH_4 emissions follow a diurnal pattern [29] and remain consistent over time when feed provision remains consistent [48]. To reduce potential timepoint measures bias and to account for diurnal variability with CH_4 production dropping dramatically overnight, average data were produced within timepoints then within time periods so that GEM visits when Asp-Oil efficacy was at its lowest would not dominate the data set. All things considered, when compared to the respiration chamber measurements it is established that in the present commercial feedlot setting that the GEM units have underestimated the extent of CH_4 inhibition. The estimates of CH_4 inhibition as defined using

GEM units should be considered the minimum potential CH_4 inhibition, with a higher confidence estimate of potential inhibition derived in the preliminary experiment to determine the optimal inclusion level [3]. That said, the GEM technique is a valuable technique for economic and real time emissions monitoring in natural and commercial environments and improved sensor options with much lower LOD are newly available (C-Lock Inc, Rapid City, South Dakota, USA). Research to confirm or differentiate GEM estimations compared to established monitoring techniques, particularly respiration chambers are warranted.

The emissions reduction range ascertained for the feedlot finisher period of 58% - 98% is a collective of accounting for the different CH_4 monitoring technologies and two outlier emitters (**Figure 4**). Contributing to differences in CH_4 reduction efficacy was an odd occurrence where two of the Asp-Oil steers were not showing reductions such that they were more analogous to the Control group than their Asp-Oil cohorts. The emissions estimates of these two steers are inexplicable as they don't conform with CH_4 reductions of the Asp-Oil group or previously reported inhibition observed with TMR inclusion of *Asparagopsis* products using respiration chambers [1] [3] [42]. As outliers the inclination is to remove them from the data pool, however, without justifiable knowledge of why these two were outliers, removing or clipping them was deemed inappropriate. Potentially they have somehow managed to avoid consuming the Asp-Oil which seems unlikely considering they were in group pens where the rest of their cohorts had the expected response. These two may have been adept at sorting the feed and selecting portions that had no Asp-Oil, which would be expected to reduce DMI, however they maintained ADWG typical of their cohorts throughout the study. If they were somehow immune to Asp-Oil $CHBr_3$ they would be expected to present a reduced efficacy with partial inhibition and not be 100% immune. Considering the GEM is a technology dependent on accurate data collection and management, although unlikely with the stringent protocols, it is possible that data for these two steers was somehow corrupted or not attributable to these two steers. To that end, **Figure 3** illustrates that H_2 emissions remained consistent and independent of including or excluding the two CH_4 emitter outliers. Typically, ruminant H_2 emissions inversely reflect CH_4 emissions [34]. Tenuously supporting the theory of corrupted data, it is expected that H_2 of the emitter outliers would be like Control H_2 which was not observed and the H_2 emissions of the Asp-Oil treatment group is not affected by including or excluding them (**Table 8**; **Figure 3**).

5. Conclusion

This research has been able to confirm, with high levels of replication and statistical power, that productivity gains are expected with high levels of CH_4 inhibition by feeding a TMR delivering *Asparagopsis* bioactives prescribed as 35 mg $CHBr_3$/kg DMI in commercial feedlots. Efficiency gains as FCE were achieved by a reduction in feed DMI, with no significant change in ADWG, HSCW, or carcass grading attributes, resulting in a lower cost of production. Furthermore, Asp-Oil

was confirmed to have no effect on food quality and safety, animal welfare, and consumer eating quality and preference. It was demonstrated that discrepancies exist in the technologies for monitoring ruminant CH_4 inhibition and further work is required to characterize the capability for protracted use, particularly for emissions accounting at scale. Overall, the confirmations of this study are expected to motivate commercial adoption of Asp-Oil and *Asparagopsis* products for feedlot beef production. Commercial adoption of *Asparagopsis* can benefit beef, sheep, and dairy production and improve the image and climate change contribution of the wider agriculture sector.

Author Contributions

Conceptualization, R.D.K., B.M.R., F.C.C.; Methodology, F.C.C., B.M.R., R.D.K., M.R.S.F., C.P., H.C., R.P.; Formal analysis, G.T., B.M.R, R.D.K, M.R.S.F., C.P., F.C.C.; Investigation, S.L.M., M.R.S.F., C.P., H.C., R.P., F.C.C.; Data curation, B.M.R., G.T., F.C.C.; Writing—original draft preparation, R.D.K., B.M.R., F.C.C.; Writing—review and editing, R.D.K., B.M.R., F.C.C., M.R.S.F., C.P., H.C., R.P., G.T.; Project administration, B.M.R., F.C.C., R.D.K.; Funding acquisition, R.D.K. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

ADWG, average daily weight gain; Asp-Meal, dried and ground *Asparagopsis* meal; Asp-Oil, *Asparagopsis* methane inhibiting bioactives stabilized in edible oil; Br^-, bromide; CH_4, methane; CH_4 Production, methane production on a grams per day basis; $CHBr_3$, bromoform; CO_2, carbon dioxide; DM, dry matter; DMI, dry matter intake; FCE, feed conversion efficiency reported as a ratio between average daily weight gain and dry matter intake; GEM, GreenFeed Emissions Monitor; [H], metabolic hydrogen; H_2, gaseous hydrogen; H_2 Production, hydrogen

production on a grams per day basis; HSCW, hot standard carcass weight; I^-, iodide; LOD, limit of detection; MSA, Meat Standards Australia; ND, non-detectable concentration of an analytical test; TMR, total mixed ration; VFAs, volatile fatty acids

Conflicts of Interest

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

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